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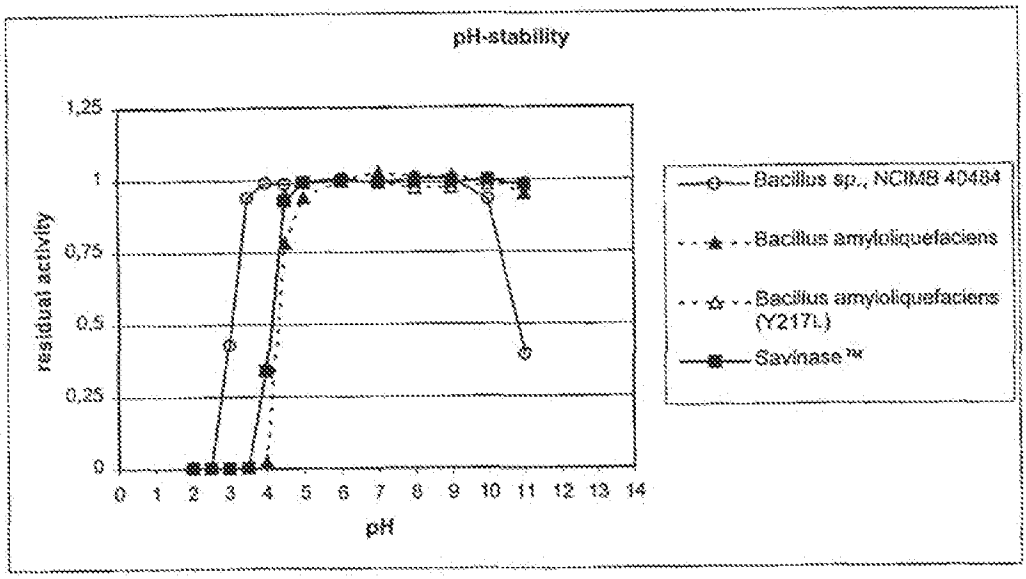
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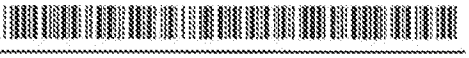
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(54) Title: USE OF ACID-STABLE SUBTILISIN PROTEASES IN ANIMAL FEED



(57) Abstract: Acid-stable proteases of the subtilisin family, their use in animal feed, feed-additives and feed compositions containing such proteases, and methods for the treatment of vegetable proteins using such proteases.

WO 01/58275 A2



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Use of Acid-stable Subtilisin Proteases in Animal Feed.

Technical Field

The present invention relates to the use of acid-stable, serine proteases of the subtilisin family in animal feed (*in vivo*), and to the use of such proteases for treating vegetable proteins (*in vitro*).

Proteins are essential nutritional factors for animals and humans. Most livestock and many human beings get the necessary proteins from vegetable protein sources. Important vegetable protein sources are e.g. oilseed crops, legumes and cereals.

When e.g. soybean meal is included in the feed of mono-gastric animals such as pigs and poultry, a significant proportion of the soybean meal solids is not digested. E.g., the apparent ileal protein digestibility in piglets and growing pigs is only around 80%.

The stomach of mono-gastric animals and many fish exhibits a strongly acidic pH. Most of the protein digestion, however, occurs in the small intestine. A need therefore exists for an acid-stable protease that can survive passage of the stomach.

Background Art

The use of proteases in animal feed, or to treat vegetable proteins, is known from the following documents:

WO95/28850 discloses i.a. an animal feed additive comprising a phytase and a proteolytic enzyme. Various proteolytic enzymes are specified at p. 7.

WO96/05739 discloses an enzyme feed additive comprising xylanase and a protease. Suitable proteases are listed at p. 25.

WO95/02044 discloses i.a. proteases derived from Aspergillus aculeatus, as well as the use in animal feed thereof.

US 3966971 discloses a process of obtaining protein from a vegetable protein source by treatment with an acid phytase

and optionally a proteolytic enzyme. Suitable proteases are specified in column 2.

US 4073884, US 5047240, US 3868448, US 3823072, and US 3683069 describe protease preparations derived from various strains of Streptomyces and their use in animal feed.

These proteases, however, are not acid-stable and/or are not proteases of the subtilisin family.

Brief Description of the Invention

Several proteases have now been identified which are found to be very acid-stable, and expectedly of an improved performance in animal feed. These proteases belong to the group of proteases known as subtilisins.

Brief Description of Drawings

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows pH-stability curves, viz. residual protease activity of four proteases (one acid-stable protease of the subtilisin family derived from Bacillus sp. NCIMB 40484 (PD 498), and three reference proteases (Sub.Novo, and Sub.Novo(Y217L), both derived from Bacillus amyloliquefaciens, and SAVINASE™) after incubation for 2 hours, at a temperature of 37°C, and at pH-values in the range of pH 2 to pH 11; the activity is relative to residual activity after a 2 hour incubation at pH 9.0, and 5°C;

Fig. 2 shows pH-activity curves, viz. protease activity between pH 3 and pH 11, relative to the protease activity at pH-optimum, of the same four proteases;

Fig. 3 shows temperature-activity curves at pH 9.0, viz. protease activity at pH 9.0 between 15°C and 80°C, relative to protease activity at the optimum temperature, of the same four proteases;

Fig. 4 shows pH-stability curves similar to Fig. 1 but for six other acid-stable proteases of the subtilisin family derived from Bacillus alcalophilus NCIMB 10438, Fusarium oxysporum IFO 4471, Paecilomyces lilacinus CBS 102449,

Aspergillus sp. CBS 102448, Acremonium chrysogenum ATCC 48272, Acremonium kiliense ATCC 20338;

Fig. 5 shows pH-activity curves similar to Fig. 2 but for the same proteases as in Fig. 4; and

Fig. 6 shows temperature activity curves at pH 9.0 similar to Fig. 3 but for the same proteases as in Fig. 4.

Detailed description of the invention

The term protease as used herein is an enzyme that hydrolyses peptide bonds (has protease activity). Proteases are also called e.g. peptidases, proteinases, peptide hydrolases, or proteolytic enzymes.

Preferred proteases for use according to the invention are of the endo-type that act internally in polypeptide chains (endopeptidases). Endopeptidases show activity on N- and C-terminally blocked peptide substrates that are relevant for the specificity of the protease in question.

Included in the above definition of protease are any enzymes belonging to the EC 3.4 enzyme group (including each of the thirteen sub-subclasses thereof) of the EC list (Enzyme Nomenclature 1992 from NC-IUBMB, 1992), as regularly supplemented and updated, see e.g. the World Wide Web (WWW) at http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html.

Proteases are classified on the basis of their catalytic mechanism into the following groupings: serine proteases (S), cysteine proteases (C), aspartic proteases (A), metalloproteases (M), and unknown, or as yet unclassified, proteases (U), see Handbook of Proteolytic Enzymes, A.J.Barrett, N.D.Rawlings, J.F.Woessner (eds), Academic Press (1998), in particular the general introduction part.

The term serine protease refers to serine peptidases and their clans as defined in the above Handbook. In the 1998 version of this handbook, serine peptidases and their clans are dealt with in chapters 1-175.

In a particular embodiment, serine proteases are peptidases in which the catalytic mechanism depends upon the hydroxyl group of a serine residue acting as the nucleophile that attacks the peptide bond.

The terms subtilisins or subtilisin family as used herein are intended to include all Clan SB serine proteases, in particular Family S8 thereof (Clan SB is dealt with in Chapter 93 of the above handbook). In subtilisins, the order of the catalytic triad is Asp-His-Ser. The tertiary structure includes both alpha-helices and beta sheets. Clan SB includes both endopeptidases and exopeptidases. These peptidases are known from bacteria, archaea and eukaryotes; there is a single representative from a DNA virus.

For determining whether a given protease is a subtilisin or not, reference is made to the above Handbook and the principles indicated therein. Such determination can be carried out for all types of proteases, be it naturally occurring or wild-type proteases; or genetically engineered or synthetic proteases.

In the alternative, inhibition studies can be performed with SSI (the Streptomyces Subtilisin Inhibitor), and a subtilisin is defined as a protease with up to 10% residual activity when inhibited with a molar excess of SSI. This test may be carried out as described in Example 8. In particular embodiments of this definition, the subtilisin has up to 8%, up to 6%, or up to 5% residual activity. The expression `up to' is considered equal to the expression `less than or equal to'.

Protease activity can be measured using any assay, in which a substrate is employed, that includes peptide bonds relevant for the specificity of the protease in question. Assay-pH and assay-temperature are likewise to be adapted to the protease in question. Examples of assay-pH-values are pH 5, 6, 7, 8, 9, 10, or 11. Examples of assay-temperatures are 25, 30, 35, 37, 40, 45, 50, 55, 60, 65, or 70°C.

Examples of protease substrates are casein, and pNA-substrates, such as Suc-AAPF-pNA (available e.g. from Sigma S-7388). The capital letters in this pNA-substrate refers to the one-letter amino acid code. Another example is Protazyme AK (azurine-dyed crosslinked casein prepared as tablets by Megazyme T-PRAK). For pH-activity and pH-stability studies,

the pNA-substrate is preferred, whereas for temperature-activity studies, the Protazyme AK substrate is preferred.

Examples of protease assays are described in the experimental part.

There are no limitations on the origin of the protease for use according to the invention. Thus, the term protease includes not only natural or wild-type proteases, but also any mutants, variants, fragments etc. thereof exhibiting protease activity, as well as synthetic proteases, such as shuffled proteases, and consensus proteases. Such genetically engineered proteases can be prepared as is generally known in the art, eg by Site-directed Mutagenesis, by PCR (using a PCR fragment containing the desired mutation as one of the primers in the PCR reactions), or by Random Mutagenesis. The preparation of consensus proteins is described in eg EP 897985.

Examples of acid-stable proteases of the subtilisin family for use according to the invention are

(i) the proteases derived from Bacillus sp. NCIMB 40484, Bacillus alcalophilus NCIMB 10438; Fusarium oxysporum IFO 4471; Paecilomyces lilacinus CBS 102449, Aspergillus sp. CBS 102448, Acremonium chrysogenum ATCC 48272, and Acremonium kiliense ATCC 20338;

(ii) proteases of at least 70, 75, 80, 85, 90, or at least 95% amino acid identity to any of the proteases of (i);

(iii) proteases of at least 70, 75, 80, 85, 90, or at least 95% identity to any of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4;

(iv) proteases of at least 70, 75, 80, 85, 90, or at least 95% amino acid identity to any of SEQ ID NO: 5 (the whole sequence 1-397, or fragments 28-397 or 118-397 thereof), SEQ ID NO: 6 (the whole sequence 1-367, or fragments 70-367 or 84-367 thereof), or SEQ ID NO: 7.

For calculating percentage identity, any computer program known in the art can be used, such as GAP provided in the GCG version 8 program package (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B.

and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

In a particular embodiment, the protease for use according to the invention is a microbial protease, the term microbial indicating that the protease is derived from, or originates from, a microorganism, or is an analogue, a fragment, a variant, a mutant, or a synthetic protease derived from a microorganism. It may be produced or expressed in the original wild-type microbial strain, in another microbial strain, or in a plant; i.e. the term covers the expression of wild-type, naturally occurring proteases, as well as expression in any host of recombinant, genetically engineered or synthetic proteases.

The term microorganism as used herein includes Archaea, bacteria, fungi, vira etc.

Examples of microorganisms are bacteria, such as bacteria of the genus Bacillus, e.g. Bacillus sp. NCIMB No.40484; Bacillus alcalophilus NCIMB 10438; or mutants or variants thereof exhibiting protease activity.

Further examples of microorganisms are fungi, such as yeast or filamentous fungi, e.g. chosen from the genera Paecilomyces, e.g. Paecilomyces lilacinus CBS 102449, Aspergillus, e.g. Aspergillus sp. CBS 102448, Acremonium, e.g. Acremonium chrysogenum ATCC 48272, Acremonium kiliense ATCC 20338, or Fusarium, e.g. Fusarium oxysporum IFO 4471; or mutants or variants thereof exhibiting protease activity.

In another embodiment the protease is a plant protease. An example of a protease of plant origin is the protease from the sarcocarp of melon fruit (Kaneda et al, J.Biochem. 78, 1287-1296 (1975).

The term animal includes all animals, including human beings. Examples of animals are non-ruminants, and ruminants, such as cows, sheep and horses. In a particular embodiment, the animal is a non-ruminant animal. Non-ruminant animals include mono-gastric animals, e.g. pigs or swine (including, but not limited to, piglets, growing pigs, and sows); poultry

such as turkeys and chicken (including but not limited to broiler chicks, layers); young calves; and fish (including but not limited to salmon).

The term feed or feed composition means any compound, preparation, mixture, or composition suitable for, or intended for intake by an animal.

In the use according to the invention the protease can be fed to the animal before, after, or simultaneously with the diet. The latter is preferred.

In the present context, the term acid-stable means, that the protease activity of the pure protease enzyme, in a dilution corresponding to A_{280} = 1.0, and following incubation for 2 hours at 37°C in the following buffer:

100mM succinic acid, 100mM HEPES, 100mM CHES,
100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 3.5,

is at least 40% of the reference activity, as measured using the assay described in Example 2C herein (substrate: Suc-AAPF-pNA, pH 9.0, 25°C).

In particular embodiments of the above acid-stability definition, the protease activity is at least 45, 50, 55, 60, 65, 70, 75, 80, 85, or at least 90% of the reference activity.

The term reference activity refers to the protease activity of the same protease, following incubation in pure form, in a dilution corresponding to A_{280} = 1.0, for 2 hours at 5°C in the following buffer: 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 9.0, wherein the activity is determined as described above.

In other words, the method of determining acid-stability comprises the following steps:

a) The protease sample to be tested (in pure form, A_{280} = 1.0) is divided in two aliquots (I and II);

b) Aliquot I is incubated for 2 hours at 37°C and pH 3.5;

c) Residual activity of aliquot I is measured (pH 9.0 and 25°C);

d) Aliquot II is incubated for 2 hours at 5°C and pH 9.0;

e) Residual activity of aliquot II is measured (pH 9.0 and 25°C);

f) Percentage residual activity of aliquot I relative to residual activity of aliquot II is calculated.

Alternatively, in the above definition of acid-stability, the step b) buffer pH-value may be 2.0, 2.5, 3.0, 3.1, 3.2, 3.3, or 3.4.

In other alternative embodiments of the above acid-stability definition relating to the above alternative step b) buffer pH-values, the residual protease activity as compared to the reference, is at least 5, 10, 15, 20, 25, 30, 35, 40, 45, or at least 50%.

In alternative embodiments, pH values of 6.0, 6.5, 7.0, 7.5, 8.0, or 8.5 can be applied for the step d) buffer.

In the above acid-stability definition, the term A_{280} = 1.0 means such concentration (dilution) of said pure protease which gives rise to an absorption of 1.0 at 280 nm in a 1cm path length cuvette relative to a buffer blank.

And in the above acid-stability definition, the term pure protease refers to a sample with a A_{280}/A_{260} ratio above or equal to 1.70 (see Example 2E), and which by a scan of a Coomassie-stained SDS-PAGE gel is measured to have at least 95% of its scan intensity in the band corresponding to said protease (see Example 2A). In the alternative, the A_{280}/A_{260} ratio is above or equal to 1.50, 1.60, 1.65, 1.70, 1.75, 1.80, 1.85, or above or equal to 1.90.

However, for the uses according to the invention, the protease need not be that pure; it may e.g. include other enzymes, even other proteases, in which case it could be termed a protease preparation. Nevertheless, a well-defined protease preparation is advantageous. For instance, it is much easier to dose correctly to the feed a protease that is essentially free from interfering or contaminating other proteases. The term dose correctly refers in particular to the objective of obtaining consistent and constant results, and

the capability of optimising dosage based upon the desired effect.

In a particular embodiment, the protease, in the form in which it is added to the feed, or when being included in a feed additive, is well-defined. Well-defined means that the protease preparation is at least 50% pure as determined by Size-exclusion chromatography (see Example 12).

In other particular embodiments the protease preparation is at least 60, 70, 80, 85, 88, 90, 92, 94, or at least 95% pure as determined by this method.

In the alternative, the term well-defined means, that a fractionation of the protease preparation on an appropriate Size-exclusion column reveals only one major protease component.

The skilled worker will know how to select an appropriate Size-exclusion chromatography column. He might start by fractionating the preparation on e.g. a HiLoad26/60 Superdex75pg column from Amersham Pharmacia Biotech (see Example 12). If the peaks would not be clearly separated he would try different columns (e.g. with an amended column particle size and/or column length), and/or he would amend the sample volume. By simple and common trial-and-error methods he would thereby arrive at a column with a sufficient resolution (clear separation of peaks), on the basis of which the purity calculation is performed as described in Example 12.

The protease preparation can be (a) added directly to the feed (or used directly in the treatment process of vegetable proteins), or (b) it can be used in the production of one or more intermediate compositions such as feed additives or premixes that is subsequently added to the feed (or used in a treatment process). The degree of purity described above refers to the purity of the original protease preparation, whether used according to (a) or (b) above.

Protease preparations with purities of this order of magnitude are in particular obtainable using recombinant methods of production, whereas they are not so easily obtained and also subject to a much higher batch-to-batch variation

when the protease is produced by traditional fermentation methods.

Such protease preparation may of course be mixed with other enzymes.

In one particular embodiment, the protease for use according to the invention, besides being acid-stable, also has a pH-activity optimum close to neutral.

The term pH-activity optimum close to neutral means one or more of the following: That the pH-optimum is in the interval of pH 6.0-11.0, or pH 7.0-11.0, or pH 6.0-10.0, or pH 7.0-10.0, or pH 8.0-11.0, or pH 8.0-10.0 (see Examples 2B and 7, and Figs. 2 and 5 herein).

In another particular embodiment, the protease for use according to the invention, besides being acid-stable, is also thermostable.

The term thermostable means one or more of the following: That the temperature optimum is at least 50°C, 52°C, 54°C, 56°C, 58°C, 60°C, 62°C, 64°C, 66°C, 68°C, or at least 70°C, reference being made to Examples 2D and 7 and Figs. 3 and 6 herein.

In a further particular embodiment, the protease for use according to the invention is capable of solubilising vegetable proteins according to the *in vitro* model of Example 4 herein.

The term vegetable proteins as used herein refers to any compound, composition, preparation or mixture that includes at least one protein derived from or originating from a vegetable, including modified proteins and protein-derivatives. In particular embodiments, the protein content of the vegetable proteins is at least 10, 20, 30, 40, 50, or 60% (w/w).

Vegetable proteins may be derived from vegetable protein sources, such as legumes and cereals, for example materials from plants of the families Fabaceae (Leguminosae), Cruciferaceae, Chenopodiaceae, and Poaceae, such as soy bean meal, lupin meal and rapeseed meal.

In a particular embodiment, the vegetable protein source is material from one or more plants of the family Fabaceae, e.g. soybean, lupine, pea, or bean.

In another particular embodiment, the vegetable protein source is material from one or more plants of the family Chenopodiaceae, e.g. beet, sugar beet, spinach or quinoa.

Other examples of vegetable protein sources are rapeseed, and cabbage.

Soybean is a preferred vegetable protein source.

Other examples of vegetable protein sources are cereals such as barley, wheat, rye, oat, maize (corn), rice, and sorghum.

The treatment according to the invention of vegetable proteins with at least one acid-stable protease of the subtilisin family results in an increased solubilisation of vegetable proteins.

The following are examples of % solubilised protein obtainable using the proteases of the invention: At least 76.8%, 77.0%, 77.2%, 77.4%, 77.6%, 77.8%, 78.0%, 78.2%, 78.4%, 78.6%, or at least 78.8%, reference being had to the *in vitro* model of Example 4 herein.

The term solubilisation of proteins basically means bringing protein(s) into solution. Such solubilisation may be due to protease-mediated release of protein from other components of the usually complex natural compositions such as feed. Solubilisation can be measured as an increase in the amount of soluble proteins, by reference to a sample with no protease treatment (see Example 4 herein).

In a particular embodiment of a treatment process the protease(s) in question is affecting (or acting on, or exerting its solubilising influence on the vegetable proteins or protein sources. To achieve this, the vegetable protein or protein source is typically suspended in a solvent, eg an aqueous solvent such as water, and the pH and temperature values are adjusted paying due regard to the characteristics of the enzyme in question. For example, the treatment may take place at a pH-value at which the relative activity of the actual protease is at least 50, or 60, or 70, or 80 or 90%.

Likewise, for example, the treatment may take place at a temperature at which the relative activity of the actual protease is at least 50, or 60, or 70, or 80 or 90% (these relative activities being defined as in Example 2 herein). The enzymatic reaction is continued until the desired result is achieved, following which it may or may not be stopped by inactivating the enzyme, e.g. by a heat-treatment step.

In another particular embodiment of a treatment process of the invention, the protease action is sustained, meaning e.g. that the protease is added to the vegetable proteins or protein sources, but its solubilising influence is so to speak not switched on until later when desired, once suitable solubilising conditions are established, or once any enzyme inhibitors are inactivated, or whatever other means could have been applied to postpone the action of the enzyme.

In one embodiment the treatment is a pre-treatment of animal feed or vegetable proteins for use in animal feed, i.e. the proteins are solubilised before intake.

The term improving the nutritional value of an animal feed means improving the availability of the proteins, thereby leading to increased protein extraction, higher protein yields, and/or improved protein utilisation. The nutritional value of the feed is therefore increased, and the growth rate and/or weight gain and/or feed conversion (i.e. the weight of ingested feed relative to weight gain) of the animal is/are improved.

In particular embodiments the weight gain is at least 101%, 102%, 103%, 104%, 105%, 106%, or at least 106.6% of the control, reference being had to Example 10 herein.

In further particular embodiments the feed conversion is at most (or not more than) 99%, 98%, 97.5%, 97%, or at most 96.6%. This is equivalent to a feed conversion of up to 99%, 98%, 97.5%, 97%, or up to 96.6%. Again, reference is had to Example 10 herein, comparing with the control.

The protease can be added to the feed in any form, be it as a relatively pure protease, or in admixture with other components intended for addition to animal feed, i.e. in the

form of animal feed additives, such as the so-called pre-mixes for animal feed.

Animal feed additives

Apart from the acid-stable protease of the subtilisin family, the animal feed additives of the invention contain at least one fat-soluble vitamin, and/or at least one water-soluble vitamin, and/or at least one trace mineral, and/or at least one macro mineral.

Further, optional, feed-additive ingredients are colouring agents, aroma compounds, stabilisers, and/or at least one other enzyme selected from amongst phytases EC 3.1.3.8 or 3.1.3.26; xylanases EC 3.2.1.8; galactanases EC 3.2.1.89; and/or beta-glucanases EC 3.2.1.4 (EC refers to Enzyme Classes according to Enzyme Nomenclature 1992 from NC-IUBMB, 1992), see also the World Wide Web (WWW) at http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html.

In a particular embodiment these other enzymes are well-defined (as defined and exemplified above for protease preparations, i.a. by reference to Example 12).

Usually fat- and water-soluble vitamins, as well as trace minerals form part of a so-called premix intended for addition to the feed, whereas macro minerals are usually separately added to the feed. Either of these composition types, when enriched with an acid-stable subtilisin according to the invention, is an animal feed additive of the invention.

In a particular embodiment, the animal feed additive of the invention is intended for being included (or prescribed as having to be included) in animal diets or feed at levels of 0.01-10.0%; more particularly 0.05-5.0%; or 0.2-1.0% (% meaning g additive per 100 g feed). This is so in particular for premixes.

Accordingly, the concentrations of the individual components of the animal feed additive, e.g. the premix, can be found by multiplying the final in-feed concentration of the same component by, respectively, 10-10000; 20-2000; or 100-500 (referring to the above three percentage inclusion intervals).

Guidelines for desired final concentrations, i.e. in-feed-concentrations, of such individual feed and feed additive components are indicated in Table A below.

The following are non-exclusive lists of examples of these components:

Examples of fat-soluble vitamins are vitamin A, vitamin D3, vitamin E, and vitamin K, e.g. vitamin K3.

Examples of water-soluble vitamins are vitamin B12, biotin and choline, vitamin B1, vitamin B2, vitamin B6, niacin, folic acid and panthothenate, e.g. Ca-D-panthothenate.

Examples of trace minerals are manganese, zinc, iron, copper, iodine, selenium, and cobalt.

Examples of macro minerals are calcium, phosphorus and sodium.

The nutritional requirements of these components - exemplified with poultry and piglets/pigs - are listed in Table A below. Nutritional requirement means that these components should be provided in the diet in the concentrations indicated. These data are compiled from:

NRC, Nutrient requirements in swine, ninth revised edition 1988, subcommittee on swine nutrition, committee on animal nutrition, board of agriculture, national research council. National Academy Press, Washington, D.C. 1988; and

NRC, Nutrient requirements of poultry, ninth revised edition 1994, subcommittee on poultry nutrition, committee on animal nutrition, board of agriculture, national research council. National Academy Press, Washington, D.C. 1994.

In the alternative, the animal feed additive of the invention comprises at least one of the individual components specified in Table A. At least one means either of, one or more of, one, or two, or three, or four and so forth up to all thirteen, or up to all fifteen individual components.

More specifically, this at least one individual component is included in the additive of the invention in such an amount as to provide an in-feed-concentration within the range indicated in column four, or column five, or column six of Table A.

As explained above, corresponding feed additive concentrations can be found by multiplying the interval limits of these ranges with 10-10000; 20-2000; or 100-500. As an example, considering which premix-content of vitamin A would correspond to the feed-content of 10-10000 IU/kg, this exercise would lead to the following intervals: 100-10^8 IU; or 200-$2x10^7$ IU; or 1000-$5x10^6$ IU per kg additive.

Table A

Nutrient requirements - and preferred ranges

Nutrients provided per kg diet	Poultry	Piglets/Pigs /Sows	Range 1	Range 2	Range 3
Fat-soluble vitamins					
Vitamin A/[IU]	~5000	1300-4000	10-10000	50-8000	100-6000
Vitamin D_3/[IU]	~1100	150-200	2-3000	5-2000	10-1500
Vitamin E/[IU]	~12	11-22	0.02-100	0.2-80	0.5-50
Vitamin K/[mg]	0.5-1.5	~0.5	0.005-10.0	0.05-5.0	0.1-3.0
Water-soluble vitamins					
B_{12}/[mg]	~0.003	0.005-0.02	0.0001-1.000	0.0005-0.500	0.001-0.100
Biotin/[mg]	0.100-0.25	0.05-0.08	0.001-10.00	0.005-5.00	0.01-1.00
Choline/[mg]	800-1600	300-600	1-10000	5-5000	10-3000
Trace minerals					
Manganese/[mg]	~60	2.0-4.0	0.1-1000	0.5-500	1.0-100
Zinc/[mg]	40-70	50-100	1-1000	5-800	10-300
Iron/[mg]	50-80	40-100	1-1000	5-500	10-300
Copper/[mg]	6-8	3.0-6.0	0.1-1000	0.5-100	1.0-25
Iodine/[mg]	~0.4	~0.14	0.01-100	0.05-10	0.1-1.0
Selenium/[mg]	~0.2	0.10-0.30	0.005-100	0.01-10.0	0.05-1.0
Macro minerals					
Calcium/[g]	8-40	5-9	0.1-200	0.5-150	1-100
Phosphorus, as available phosphorus/[g]	3-6	1.5-6	0.1-200	0.5-150	1-50

Animal feed compositions

Animal feed compositions or diets have a relatively high content of protein. According to the National Research Council

(NRC) publications referred to above, poultry and pig diets can be characterised as indicated in Table B below, columns 2-3. Fish diets can be characterised as indicated in column 4 of Table B. Furthermore such fish diets usually have a crude fat content of 200-310 g/kg. These fish diet are exemplified with diets for Salmonids and designed on the basis of Aquaculture, principles and practices, ed. T.V.R. Pillay, Blackwell Scientific Publications Ltd. 1990; Fish nutrition, second edition, ed. John E. Halver, Academic Press Inc. 1989.

An animal feed composition according to the invention has a crude protein content of 50-800 g/kg, and furthermore comprises at least one protease as claimed herein.

Furthermore, or in the alternative (to the crude protein content indicated above), the animal feed composition of the invention has a content of metabolisable energy of 10-30 MJ/kg; and/or a content of calcium of 0.1-200 g/kg; and/or a content of available phosphorus of 0.1-200 g/kg; and/or a content of methionine of 0.1-100 g/kg; and/or a content of methionine plus cysteine of 0.1-150 g/kg; and/or a content of lysine of 0.5-50 g/kg.

In particular embodiments, the content of metabolisable energy, crude protein, calcium, phosphorus, methionine, methionine plus cysteine, and/or lysine is within any one of ranges 2, 3, 4 or 5 in Table B below (R. 2-5).

Crude protein is calculated as nitrogen (N) multiplied by a factor 6.25, i.e. Crude protein (g/kg) = N (g/kg) x 6.25 as stated in Animal Nutrition, 4th edition, Chapter 13 (Eds. P. McDonald, R. A. Edwards and J. F. D. Greenhalgh, Longman Scientific and Technical, 1988, ISBN 0-582-40903-9). The nitrogen content is determined by the Kjeldahl method (A.O.A.C., 1984, Official Methods of Analysis 14th ed., Association of Official Analytical Chemists, Washington DC).

Metabolisable energy can be calculated on the basis of the NRC publication Nutrient Requirements of Swine (1988) pp. 2-6, and the European Table of Energy Values for Poultry Feedstuffs, Spelderholt centre for poultry research and extension, 7361 DA Beekbergen, The Netherlands. Grafisch bedrijf Ponsen & looijen bv, Wageningen. ISBN 90-71463-12-5.

The dietary content of calcium, available phosphorus and amino acids in complete animal diets is calculated on the basis of feed tables such as Veevoedertabel 1997, gegevens over chemische samenstelling, verteerbaarheid en voederwaarde van voedermiddelen, Central Veevoederbureau, Runderweg 6, 8219 pk Lelystad. ISBN 90-72839-13-7.

In a particular embodiment, the animal feed composition of the invention containe at least one vegetable protein or protein source as defined above.

In still further particular embodiments, the animal feed composition of the invention contains 0-80% maize; and/or 0-80% sorghum; and/or 0-70% wheat; and/or 0-70% Barley; and/or 0-30% oats; and/or 0-40% soybean meal; and/or 0-10% fish meal; and/or 0-20% whey.

Animal diets can e.g. be manufactured as mash feed (non-pelleted) or pelleted feed. Typically, the milled feedstuffs are mixed and sufficient amounts of essential vitamins and minerals are added according to the specifications for the species in question. Enzymes can be added as solid or liquid enzyme formulations. For example, a solid enzyme formulation is typically added before or during the mixing step; and a liquid enzyme preparation is typically added after the pelleting step. The enzyme may also be incorporated in a feed additive or premix. The final enzyme concentration in the diet is within the range of 0.01-200 mg enzyme protein per kg diet, for example in the range of 5-30 mg enzyme protein per kg animal diet.

Examples of animal feed compositions are shown in Example 11.

Table B
Range values for energy, protein and minerals in animal diets

Nutrient	Poultry	Piglets/Pigs/Sows	Fish	R. 1	R. 2	R. 3	R. 4	R. 5
	Min - Max	Min - Max	Min - Max					
Metabolisable energy, MJ/kg	12.1 - 13.4	12.9-13.5	14-25	10-30	11-28	11-26	12-25	
Crude protein, g/kg	124-280	120-240	300-480	50-800	75-700	100-600	110-500	120-490
Calcium, g/kg	8-40	5-9	10-15	0.1-200	0.5-150	1-100	4-50	
Available Phosphorus, g/kg	2.1-6.0	1.5-5.5	3-12	0.1-200	0.5-150	1-100	1-50	1-25
Methionine, g/kg	3.2 -5.5	-	12-16	0.1-100	0.5-75	1-50	1-30	
Methionine plus Cysteine, g/kg	4-9	2.3-6.8	-	0.1-150	0.5-125	1-80		
Lysine, g/kg	2.5-11	6-14	12-22	0.5-50	0.5-40	1-30		

In particular embodiments of the method of the invention for treating vegetable proteins, a further step of adding phytase is also included. And in further particular embodiments, in addition to the combined treatment with phytase and protease, further enzymes may also be added, wherein these enzymes are selected from the group comprising other proteases, phytases, lipolytic enzymes, and glucosidase/carbohydrase enzymes. Examples of such enzymes are indicated in WO95/28850.

The protease should of course be applied in an effective amount, i.e. in an amount adequate for improving solubilisation and/or improving nutritional value of feed. It

is at present contemplated that the enzyme is administered in one or more of the following amounts (dosage ranges): 0.01-200; or 0.01-100; or 0.05-100; or 0.05-50; or 0.10-10 - all these ranges being in mg protease protein per kg feed (ppm).

For determining mg protease protein per kg feed, the protease is purified from the feed composition, and the specific activity of the purified protease is determined using a relevant assay (see under protease activity, substrates, and assays). The protease activity of the feed composition as such is also determined using the same assay, and on the basis of these two determinations, the dosage in mg protease protein per kg feed is calculated.

The same principles apply for determining mg protease protein in feed additives.

Of course, if a sample is available of the protease used for preparing the feed additive or the feed, the specific activity is determined from this sample (no need to purify the protease from the feed composition or the additive).

Many vegetables contain anti-nutritional factors such as lectins and trypsin inhibitors. The most important anti-nutritional factors of soybean are the lectin soybean agglutinin (SBA), and the soybean trypsin inhibitor (STI).

Lectins are proteins that bind to specific carbohydrate-containing molecules with considerable specificity, and when ingested they become bound to the intestinal epithelium. This may lead to reduced viability of the epithelial cells and reduced absorption of nutrients.

SBA is a glycosylated, tetrameric lectin with a subunit molecular weight of about 30 kDa and a high affinity for N-acetylgalactosamine.

Trypsin inhibitors affect the intestinal proteolysis reducing protein digestibility, and also increase the secretion of digestive enzymes from the pancreas leading to a loss of amino acids in the form of digestive enzymes. An example of a trypsin inhibitor is the Bowman-Birk Inhibitor, that has a molecular weight of about 8 kDa, contains 7 disulfide bridges and has two inhibitor/ loops specific for trypsin-like and chymotrypsin-like proteases. Other examples

are the so-called Kunitz Inhibitors of Factors (e.g. the Soybean Kunitz Trypsin Inhibitor that contains one binding site for trypsin-like proteases and has a molecular weight of about 20 kDa).

The proteases for use according to the invention have been shown to hydrolyse anti-nutritional factors like SBA lectin, and the trypsin inhibitors Bowman Birk Inhibitor and The Soybean Kunitz Factor. See the experimental part, Example 5.

Thus, the invention also relates to the use of acid-stable serine proteases for hydrolysing, or reducing the amount of, anti-nutritional factors, e.g. SBA lectin, and trypsin inhibitors, such as the Bowman Birk Inhibitor, and Kunitz Factors, such as the Soybean Kunitz Factor.

Example 1

Screening for acid-stable proteases

A large number of proteases were analysed for stability at pH 3, with the objective of identifying proteases that have the necessary stability to pass through the acidic stomach of mono-gastric animals.

The proteases had been purified by conventional chromatographic methods such as ion-exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography (see e.g. Protein Purification, Principles, High Resolution Methods, and Applications. Editors: Jan-Christer Janson, Lars Rydén, VCH Publishers, 1989).

Protease activity was determined as follows: The protease was incubated with 1.67% Hammarsten casein at 25°C, pH 9.5 for 30 minutes, then TCA (tri-chloro acetic acid) was added to a final concentration of 2% (w/w), the mixture was filtrated to remove the sediment, and the filtrate was analysed for free primary amino groups (determined in a colometric assay based on OPA (o-phthal-dialdehyde) by measuring the absorbance at 340nm, using a serine standard (Biochemische Taschenbuch teil II, Springer-Verlag (1964), p.93 and p.102). One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1mmol of TCA-soluble primary

amino groups per minute under standard conditions, i.e. 25°C and pH 9.5.

The proteases were diluted to an activity of 0.6 CPU/l in water, divided in two aliquots and each aliquot was then further diluted to 0.3 CPU/l with 100 mM citrate buffer, pH 3, and 100 mM phosphate buffer, pH 7 respectively. The diluted samples were incubated at 37°C for 1 hour, and 20 µl of the samples were applied to holes in 1% agarose plates containing 1% skim milk. The plates (pH 7.0) were incubated at 37°C over night and clearing zones were measured.

42 proteases performed well in this test. A number of these have been characterised, see examples 2, 6, 7 and 8. These proteases all belong to the subtilisin family of serine proteases.

Example 2

Characterisation and comparative study of the subtilisin protease derived from Bacillus sp. NCIMB 40484

The protease derived from Bacillus sp. NCIMB 40484 was prepared as described in Example 1 of WO93/24623.

The purpose of this characterisation was to study its pH-stability, pH-activity and temperature-activity profiles, in comparison to Sub.Novo, Sub.Novo(Y217L), and SAVINASE™.

Sub.Novo is subtilisin from Bacillus amyloliquefaciens, and Sub.Novo(Y217L) is the mutant thereof that is disclosed in WO96/05739. Sub.Novo was prepared and purified from a culture of the wild-type strain using conventional methods, whereas the mutant was prepared as described in Examples 1-2, and 15-16 of EP 130756.

SAVINASE™ is a subtilisin derived from Bacillus clausii (previously Bacillus lentus NCIB 10309), commercially available from Novozymes A/S, Krogshoejvej, DK-2880 Bagsvaerd, Denmark. Its preparation is described in US patent No. 3723250.

Example 2A

Determination of SDS-PAGE purity of protease samples

The SDS-PAGE purity of the protease samples was determined by the following procedure:

40µl protease solution (A_{280} concentration = 0.025) was mixed with 10µl 50%(w/v) TCA (trichloroacetic acid) in an Eppendorf tube on ice. After half an hour on ice the tube was centrifuged (5 minutes, 0°C, 14.000 x g) and the supernatant was carefully removed. 20µl SDS-PAGE sample buffer (200µl Tris-Glycine SDS Sample Buffer (2x) (125mM Tris/HCl, pH 6.8, 4%(w/v) SDS, 50ppm bromophenol blue, 20%(v/v) Glycerol, LC2676 from NOVEX™) + 160µl dist. water + 20µl ß-mercaptoethanol + 20µl 3M unbuffered Tris Base (Sigma T-1503) was added to the precipitate and the tube was boiled for 3 minutes. The tube was centrifuged shortly and 10µl sample was applied to a 4-20% gradient Tris-Glycine precast gel from NOVEX™ (polyacrylamide gradient gel based on the Laemmli chemistry but without SDS in the gel, (Laemmli, U.K., (1970) Nature, vol. 227, pp. 680-685), EC60255). The electrophoresis was performed with Tris-Glycine running buffer (2.9g Tris Base, 14.4g Glycine, 1.0g SDS, distilled water to 1 liter) in both buffer reservoirs at a 150V constant voltage until the bromophenol blue tracking dye had reached the bottom of the gel. After electrophoresis, the gel was rinsed 3 times, 5 minutes each, with 100 ml of distilled water by gentle shaking. The gel was then gently shaked with Gelcode® Blue Stain Reagent (colloidal Comassie G-250 product from PIERCE, PIERCE cat. No. 24592) for one hour and washed by gentle shaking for 8 to 16 hours with distilled water with several changes of distilled water. Finally, the gel was dried between 2 pieces of cellophane. Dried gels were scanned with a Arcus II scanner from AGFA equipped with Fotolook 95 v2.08 software and imported to the image evaluation software CREAM™ for Windows (catalogue nos. 990001 and 990005, Kem-En-Tec, Denmark) by the *File/Acquire* command with the following settings (of Fotolook 95 v2.08): Original=Reflective, Mode=Color RGB, Scan resolution=240 ppi, Output resolu-

tion=120lpi, Scale factor=100%, Range=Histogram with Global selection and Min=0 and Max=215, ToneCurve=None, Sharpness=None, Descreen=None and Flavor=None, thereby producing an *.img picture file of the SDS-PAGE gel, which was used for evaluation in CREAM™. The *.img picture file was evaluated with the menu command *Analysis/1-D*. Two scan lines were placed on the *.img picture file with the *Lane Place Tool*: A Sample scan line and a Background scan line. The Sample scan line was placed in the middle of a sample lane (with the protease in question) from just below the application slot to just above the position of the Bromphenol blue tracking dye. The Background scan line was placed parallel to the Sample scan line, but at a position in the pictured SDS-PAGE gel where no sample was applied, start and endpoints for the Background scan line were perpendicular to the start and endpoints of the Sample scan line. The Background scan line represents the true background of the gel. The width and shape of the scan lines were not adjusted. The intensity along the scan lines where now recorded with the *1-D/Scan* menu command with *Medium* sensitivity. Using the *1-D/Editor* menu command, the Background scan was subtracted from the Sample scan. Then the *1-D/Results* menu command was selected and the *Area %* of the protease peak, as calculated by the CREAM™ software, was used as the SDS-PAGE purity of the proteases.

The following results were obtained:

Protease	SDS-PAGE Purity (Area %)
From Bacillus sp. NCIMB 40484	96.3
Sub.Novo	95.5
Sub.Novo (Y217L)	96.0
Savinase®	99.2

Example 2B

pH-activity assay

Suc-AAPF-pNA (Sigma® S-7388) was used for obtaining pH-activity profiles.

Assay buffer: 100mM succinic acid (Merck 1.00682), 100mM HEPES (Sigma H-3375), 100mM CHES (Sigma C-2885), 100mM CABS (Sigma C-5580), 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, adjusted to pH-values 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, or 11.0 with HCl or NaOH.

Assay temperature: 25°C.

A 300µl protease sample (diluted in 0.01% Triton® X-100) was mixed with 1.5 ml of the assay buffer at the respective pH value, bringing the pH of the mixture to the pH of the assay buffer. The reaction was started by adding 1.5ml pNA substrate (60mg dissolved in 1.0ml DMSO and further diluted 45x with 0.01% Triton® X-100) and, after mixing, the increase in A_{405} was monitored by a spectrophotometer as a measurement of the protease activity at the pH in question. The assay was repeated with the assay buffer at the other pH values, and the activity measurements were plotted as relative activity against pH. The relative activities were normalized with the highest activity (pH-optimum), i.e. setting activity at pH-optimum to 1, or to 100%. The protease samples were diluted to ensure that all activity measurements fell within the linear part of the dose-response curve for the assay.

Example 2C

pH-stability assay

Suc-AAPF-pNA (Sigma® S-7388) was used for obtaining pH-stability profiles.

Assay buffer: 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100 adjusted to pH-values 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 or 11.0 with HCl or NaOH.

Each protease sample (in 1mM succinic acid, 2mM $CaCl_2$, 100mM NaCl, pH 6.0 and with an A_{280} absorption > 10) was diluted in the assay buffer at each pH value tested to A_{280} = 1.0. The diluted protease samples were incubated for 2 hours at 37°C. After incubation, protease samples were diluted in 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 9.0, bringing the pH of all samples to pH 9.0.

In the following activity measurement, the temperature was 25°C.

300µl diluted protease sample was mixed with 1.5ml of the pH 9.0 assay buffer and the activity reaction was started by adding 1.5ml pNA substrate (50mg dissolved in 1.0ml DMSO and further diluted 45x with 0.01% Triton® X-100) and, after mixing, the increase in A_{405} was monitored by a spectrophotometer as a measurement of the (residual) protease activity. The 37°C incubation was performed at the different pH-values and the activity measurements were plotted as residual activities against pH. The residual activities were normalized with the activity of a parallel incubation (control), where the protease was diluted to A_{280} = 1.0 in the assay buffer at pH 9.0 and incubated for 2 hours at 5°C before activity measurement as the other incubations. The protease samples were diluted prior to the activity measurement in order to ensure that all activity measurements fell within the linear part of the dose-response curve for the assay.

Example 2D

Temperature-activity assay

Protazyme AK tablets were used for obtaining temperature profiles. Protazyme AK tablets are azurine dyed crosslinked casein prepared as tablets by Megazyme.

Assay buffer: 100mM succinic acid, 100mM HEPBS, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100 adjusted to pH 9.0 with NaOH.

A Protazyme AK tablet was suspended in 2.0ml 0.01% Triton® X-100 by gentle stirring. 500µl of this suspension and 500µl assay buffer were mixed in an Eppendorf tube and placed on ice. 20µl protease sample (diluted in 0.01% Triton X-100) was added. The assay was initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate. By transferring the tube back to the ice bath, the assay incubation was stopped. The tube was centrifuged in an ice-cold centrifuge for a few minutes and the A_{650} of the supernatant was read by a spectrophotometer. A buffer blind was included in the assay (instead of enzyme). A_{650}(protease) - A_{650}(blind) was a measurement of protease activity. The assay was performed at different temperatures and the activity measurements were plotted as relative activities against incubation temperature. The relative activities were normalized with the highest activity (temperature optimum). The protease samples were diluted to ensure that all activity measurements fell within the near linear part of the dose-response curve for the assay.

An overview of the activity optima (pH- and temperature activity) is seen in Table 1. pH-stability, pH-activity and temperature-activity profiles are seen in figures 1-3, and a detailed comparison of the pH-stability data for the proteases at acidic pH-values is seen in Table 2.

Table 1

pH- and temperature optima of various proteases

Protease	pH-optimum (pNA-substrate)	Temperature-optimum at pH 9.0 (Protazyme AK)
From Bacillus sp. NCIMB 40484	9	60°C
Sub.Novo*	10	70°C
Sub.Novo(Y217L)*	9	70°C
SAVINASE™)	9	70°C

Table 2

pH-stability of various proteases, between pH 2.0 and 5.0

Protease	pH 2.0	pH 2.5	pH 3.0	pH 3.5	pH 4.0	pH 4.5	pH 5.0
From Bacillus sp. NCIMB 40484	0.001	0.001	0.428	0.940	0.991	0.989	0.991
Sub.Novo	0.007	0.003	0.000	0.000	0.024	0.784	0.942
Sub.Novo(Y217L)	0.000	0.000	0.002	0.003	0.350	0.951	0.996
Savinase	0.001	0.001	0.001	0.003	0.338	0.929	0.992

Example 2E

Absorption purity of purified protease samples

Determination of A_{280}/A_{260} ratio

The A_{280}/A_{260} ratio of purified protease samples is determined as follows.

A_{260} means the absorption of a protease sample at 260 nm in a 1cm path length cuvette relative to a buffer blank. A_{280} means the absorption of the same protease sample at 280 nm in a 1cm path length cuvette relative to a buffer blank.

Samples of the purified proteases from Examples 2 and 6 were diluted in buffer until the A_{280} reading of the spectrophotometer was within the linear part of its response curve. The A_{280}/A_{260} ratio was determined from the readings.

The following results were obtained:

Protease / subtilisin from	A_{280}/A_{260}
Sub.Novo	2.11
Sub.Novo(Y217L)	2.12
SAVINASE™	2.12
Bacillus sp., NCIMB 40484	2.19
Bacillus alcalophilus, NCIMB 10438	1.92
Fusarium oxysporum, IFO 4471	1.89
Paecilomyces lilacinus, CBS 102449	1.92
Aspergillus sp., CBS 102448	1.96
Acremonium chrysogenum, ATCC 48272	2.04
Acremonium kiliense, ATCC 20338	1.71

Example 3

Ability of the protease derived from Bacillus sp. NCIMB 40484 to degrade insoluble parts of Soy Bean Meal (SBM)

The protease from Bacillus sp. NCIMB 40484 was tested for its ability to make the insoluble/indigestible parts of SBM accessible to digestive enzymes and/or added exogeneous enzymes.

Its performance was compared to two aspartate proteases, Protease I and Protease II, prepared as described in WO 95/02044. This document also discloses their use in feed. Protease I is an Aspergillopepsin II type of protease, and Protease II an Aspergillopepsin I type of protease (both aspartate proteases, ie non-subtilisin proteases) from Aspergillus aculeatus (reference being made to Handbook of Proteolytic Enzymes referred to above).

The test substrate, the so-called soy remnant, was produced in a process which mimics the digestive tract of mono-gastric animals, including a pepsin treatment at pH 2, and a pancreatin treatment at pH 7.

In the pancreatin treatment step a range of commercial enzymes was added in high dosages in order to degrade the SBM components that are accessible to existing commercial enzymes.

The following enzymes, all commercially available from Novozymes A/S, Denmark, were added: ALCALASE™ 2.4L, NEUTRASE™ 0.5L, FLAVOURZYME™ 1000L, ENERGEX™ L, BIOFEED™ Plus L,

PHYTASE NOVO™ L. The SBM used was a standard 48% protein SBM for feed, which had been pelletized.

After the treatment only 5% of the total protein was left in the resulting soy remnant.

FITC labelling protocol

The remnant was subsequently labelled with FITC (Molecular Probes, F-143) as follows: Soy remnant (25 g wet, ~ 5 g dry) was suspended in 100 ml 0.1M carbonate buffer, pH 9 and stirred 1 hour at 40°C. The suspension was cooled to room temperature and treated with fluorescein 5-isothiocyanate (FITC) over night in the dark. Non-coupled probe was removed by ultrafiltration (10.000 Mw cut-off).

FITC-assay

The FITC-labelled soy remnant was used for testing the ability of the proteases to degrade the soy remnant using the following assay: 0.4 ml protease sample (with A_{280} = 0.1) was mixed with 0.4 ml FITC-soy remnant (suspension of 10 mg/ml in 0.2M sodium-phosphate buffer pH 6.5) at 37°C, and the relative fluorescence units (RFU 485/535nm; excitation/monitoring wave length) measured after 0 hours, and after 22 hours incubation. Before determination of the RFU, samples were centrifuged for 1 min at 20.000 x G and 250 micro-liter supernatant was transferred to a black micro-titer tray. Measurements were performed using a VICTOR 1420 Multilabel counter (*In vitro*, Denmark). RFU is generally described by Iain D. Johnson in: Introduction to Fluorescence Techniques, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Richard P. Haugland, 6th edition, 1996 (ISBN 0-9652240-0-7).

A blind sample was prepared by adding 0.4 ml buffer instead of enzyme sample.

$RFU_{sample} = \Delta RFU_{sample} - \Delta RFU_{blind}$, where ΔRFU = RFU(22 hours) - RFU(0 hours)

The resulting FITC values (RFU_{sample} values) are shown in Table 3 below. The FITC values are generally with an error margin of +/- 20.000. Contrary to Protease I and Protease II, the protease derived from Bacillus sp. NCIMB 40484 degraded the soy remnant to a significant extent.

Table 3

Ability of proteases to degrade soy remnant

Protease	FITC (+/-20000)
Bacillus sp. NCIMB 40484	61900
Protease I	-9200
Protease II	-1200

Example 4

In vitro testing of the protease derived from Bacillus sp. NCIMB 40484

The protease derived from Bacillus sp. NCIMB 40484 was tested together with other subtilisin proteases such as Sub-Novo, SubNovo (Y217L), SAVINASE™ and ALCALASE™ for its ability to solubilise maize-SBM (maize-Soy Bean Meal) proteins in an automated *in vitro* digestion system (simulating digestion in monogastric animals). For the blank treatments, maize-SBM was incubated in the absence of exogenous subtilisin-like proteases.

The *in vitro* system consisted of 30 flasks in which maize-SBM substrate was initially incubated with HCl/pepsin - simulating gastric digestion - and subsequently with pancreatin - simulating intestinal digestion. At the end of the gastric incubation period samples of *in vitro* digesta were removed and analysed for solubilised protein.

Substrates

10 g maize-SBM diet with a maize-SBM ratio of 6:4 (w/w) was used. The protein content was 43% (w/w) in SBM and 8.2% (w/w) in maize meal. The total amount of protein in 10 g maize-SBM diet was 2.21 g.

Digestive enzymes

Pepsin (Sigma P-7000; 539 U/mg, solid), pancreatin (Sigma P-7545; 8xU.S.P. (US Pharmacopeia)).

Outline of *in vitro* digestion procedure

Components added to flask	pH	Temp.	Time course	Simulated digestion phase
10 g maize-SBM diet (6:4), HCl/pepsin (3000 U/g diet), protease (0.1 mg protease enzyme protein/g diet)	3.0	40°C	t=0 min	Gastric
NaOH	6.8	40°C	t=60 min	Intestinal
$NaHCO_3$/pancreatin (8 mg/g diet)	6.8	40°C	t=90 min	
Stop incubation, remove aliquot	7.0	0°C	t=330 min	

Enzyme protein determinations

The amount of protease enzyme protein is calculated on the basis of the A_{280} values and the amino acid sequences (amino acid compositions) using the principles outlined in S.C.Gill & P.H. von Hippel, Analytical Biochemistry 182, 319-326, (1989).

Experimental procedure for *in vitro* model

1. 10 g of substrate is weighed into a 100 ml flask.

2. At time 0 min, 46 ml HCl (0.1 M) containing pepsin (3000 U/g diet) and 1 ml of protease (0.1 mg enzyme protein/g diet) are added to the flask while mixing. The flask is incubated at 40°C.

3. At time 30 min, pH is measured.

4. At time 45 min, 16 ml of H_2O is added.

5. At time 60 min, 7 ml of NaOH (0.39 M) is added.

6. At time 90 min, 5 ml of $NaHCO_3$ (1M) containing pancreatin (8.0 mg/g diet) is added.

7. At time 120 min, pH is measured.

8. At time 300 min, pH is measured.

9. At time 330 min, samples of 30 ml are removed and placed on ice before centrifugation (10000 x g, 10 min, 4°C). Supernatants are removed and stored at -20°C.

Estimation of solubilised protein by gelfiltration HPLC

The content of solubilised protein in supernatants from *in-vitro* digested samples was estimated by quantifying crude protein (CP) using gelfiltration HPLC. Supernatants were thawed, filtered through 0.45 µm polycarbonate filters (Sartorius) and diluted (1:50, v/v) with H_2O. Diluted samples were chromatographed by HPLC using a Superdex Peptide PE (7.5 x 300 mm) gelfiltration column (Global). The eluent used for isocratic elution was 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. The total volume of eluent per run was 26 ml and the flow rate was 0.4 ml/min. Elution profiles were recorded at 214 nm and the total area under the profiles was determined by integration. To estimate protein content from integrated areas, a calibration curve (R^2=0.9993) was made from a dilution series of an *in vitro* digested reference maize-SBM sample with known total protein content. The protein determination in this reference sample was carried out by the Kjeldahl method (determination of % nitrogen; A.O.A.C. (1984) Official Methods of Analysis 14th ed., Washington DC).

Results

The results, i.e. the effect of the various proteases on protein solubility *in vitro*, are shown in Table 4 below.

The calculation of relative amounts of solubilised protein is based on the total amount of protein in 10 g maize-SBM diet (2.21 g protein) dissolved in a total volume of 75 ml during the *in vitro* digestion reaction. Assuming complete protein solubilisation (100%), the protein content in supernatants would be 2.95% weight per volume.

The results were analysed by one-way analysis of variance: P =0.0001). SD = Standard Deviation; n = the number of replicas per treatment (n=5).

The protease derived from Bacillus sp. NCIMB 40484 has a significantly better effect on protein solubilisation as compared to the other proteases.

Table 4

Enzyme	Soluble CP (% of total)	SD
Protease from Bacillus sp. NCIMB 40484	78.6[A]	0.48
Sub.Novo	76.7[B]	0.37
ALCALASE™	73.9[C]	1.04
Sub.Novo (Y217L)	75.8[B]	0.91
SAVINASE™	75.8[B]	0.85
Blank	76.6[B]	0.88

A, B, C: Values not sharing a common index letter differ significantly ($P < 0.05$)

Example 5

Degradation of the lectin SBA and the soybean Bowman-Birk and Kunitz Inhibitors

The ability of the protease from Bacillus sp. NCIMB 40484 to hydrolyse soybean agglutinin (SBA) and the soy Bowman-Birk and Kunitz trypsin inhibitors was tested.

Pure SBA (Fluka 61763), Bowman-Birk Inhibitor (Sigma T-9777) or Kunitz Inhibitor (Trypsin Inhibitor from soybean, Boehringer Mannheim 109886) was incubated with the protease for 2 hours, 37°C, at pH 6.5 (protease: anti-nutritional factor = 1:10, based on A_{280}). Incubation buffer: 50 mM dimethyl glutaric acid, 150 mM NaCl, 1 mM $CaCl_2$, 0.01% Triton X-100, pH 6.5.

The ability of the protease to degrade SBA and the protease inhibitors was estimated from the disappearance of the native SBA or trypsin inhibitor bands and appearance of low molecular weight degradation products on SDS-PAGE gels. Gels were stained with Coomassie blue and band intensity determined by scanning.

The results, as % of anti-nutritional factor degraded, are shown in Table 5 below.

It is contemplated that the ability to degrade the anti-nutritional factors in soy can also be estimated by applying the Western technique with antibodies against SBA, Bowman-Birk

Inhibitor or Kunitz Inhibitor after incubation of soybean meal with the candidate proteases (see WO98/56260).

Table 5

Protease derived from	SBA	Bowman-Birk Inhibitor	Kunitz Inhibitor
Bacillus sp. NCIMB 40484	21	41	100

Example 6

Preparation of further acid-stable subtilisins

Preparation of the Bacillus alcalophilus protease

Bacillus alcalophilus NCIMB 10438 was inoculated from a freeze dried culture into shake flasks each containing 100 ml BPX medium with the following composition: potato starch 100 g/l, barley flour 50 g/l, BAN 800 MG (obtainable from Novozymes A/S) 0.05 g/l, sodium caseinate 10 g/l, soy meal 20 g/l, di-sodiumphosphate 9 g/l, Pluronic PE 6100 0.1 ml/l in tap water. The pH was adjusted to 9.7 with 10 ml 1M sodium sesquicarbonate in each shake flask before inoculation. The strain was fermented for 4 days at 30 degree C at 300 rpm. From this culture new shake flasks containing 100 ml BPX medium were inoculated and fermented for 3 days.

Purification

The culture broth was centrifuged at 10000 x g for 30 minutes in 1 liter beakers. The supernatants were combined and further clarified by a filtration though a Seitz K-250 depth filter plate. The clear filtrate was concentrated by ultrafiltration on a 3kDa cut-off polyether sulfone cassette (Filtron). The concentrated enzyme was transferred to 50mM H_3BO_3, 5mM 3,3'-dimethyl glutaric acid, 1mM $CaCl_2$, pH 7 (Buffer A) on a G25 Sephadex column (Amersham Pharmacia Biotech), and applied to a Bacitracin agarose column (Upfront Chromatography A/S) equilibrated in Buffer A. After washing the Bacitracin column with Buffer A to remove unbound protein, the protease was eluted from the column using Buffer A supplemented with 25% 2-propanol and 1M sodium chloride. The fractions from the Bacitracin column with protease activity were pooled and transferred to 20mM CH_3COOH/NaOH, 1mM $CaCl_2$, pH 5 (Buffer B) by

G25 Sephadex chromatography. The buffer exchanged protease pool was applied to a SOURCE 30S column (Amersham Pharmacia Biotech) equilibrated in Buffer B. After washing the SOURCE 30S column with Buffer B, the protease was eluted with an increasing linear NaCl gradient (0 to 0.5M) in Buffer B. Fractions from the column were tested for protease activity and protease containing fractions were analysed by SDS-PAGE. Pure fractions were pooled and used for further characterisation.

Preparation of other acid-stable subtilisins

The proteases of Fusarium oxysporum IFO 4471, Bacillus alcalophilus NCIMB 10438, Paecilomyces lilacinus CBS 102449, Aspergillus sp. CBS 102448, Acremonium chrysogenum ATCC 48272, and Acremonium kiliense ATCC 20388 were prepared using conventional methods, as generally described above for the protease of Bacillus alcalophilus, NCIMB 10438.

Sequences

The following partial amino acid sequences were determined:

SEQ ID NO: 1

N-terminal of the protease derived from Acremonium chrysogenum ATCC 48272: ALVTQNGAPWGLGTISHRQPGSTSYIY;

SEQ ID NO: 2

N-terminal of the protease derived from Bacillus alcalophilus NCIMB 10438: NQVTPWGITRVQAPTAW;

SEQ ID NO: 3

N-terminal of the protease derived from Paecilomyces lilacinus CBS 102449: AYTQQPGAPWGLGRISH;

SEQ ID NO: 4

N-terminal of the protease derived from Fusarium oxysporum IFO 4471: ALTTQSGATWGLGTVSHRSRGS.

The amino acid sequence of the protease derived from Bacillus sp. NCIMB 40484, SEQ ID NO: 5 herein, had been previously determined (see US patent no. 5,650,326, SEQ ID NOs: 4, 6 and 8).

A search in public protein databases for related sequences revealed the following:

SEQ ID NO: 6

Geneseqp/r65936 (referring to the protease of Paecilomyces lilacinus CBS 143.75 of EP 623672) - related to SEQ ID NO: 3;

SEQ ID NO: 7

Geneseqp/r74334 (referring to the protease of Bacillus sp. THS-1001 of JP-07095882) - related to SEQ ID NO: 2.

The strains of Paecilomyces lilacinus and Aspergillus sp. have been deposited according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Centraalbureau voor Schimmelcultures (CBS), P.O Box 273, 3740 AG Baarn, The Netherlands, as follows.

Deposit date	:	17 January 2000
CBS No.	:	Aspergillus sp. 102448
Deposit date	:	17 January 2000
CBS No.	:	Paecilomyces lilacinus 102449

The deposits were made by Novo Nordisk A/S and were later assigned to Hoffmann-La Roche AG.

Example 7

Characterisation and comparative study of further subtilisin proteases

The proteases prepared from Bacillus alcalophilus NCIMB 10438, Fusarium oxysporum IFO 4471, Paecilomyces lilacinus CBS 102449, Aspergillus sp. CBS 102448, Acremonium chrysogenum ATCC 48272, Acremonium kiliense ATCC 20338 are all subtilisins.

The purity of the protease samples was determined as described in example 2.

The following results were obtained:

Protease	SDS-PAGE Purity (Area %)
Bacillus alcalophilus NCIMB 10438	100.0
Fusarium oxysporum IFO 4471	n.d.
Paecilomyces lilacinus CBS 102449	98.3
Aspergillus sp. CBS 102448	n.d.
Acremonium chrysogenum ATCC 48272	98.6
Acremonium kiliense ATCC 20338	n.d.

n.d. = not determined

Assays

The pH-activity, pH-stability and temperature-activity assays are described in Example 2 (the pNA substrate Suc-AAPF-pNA (Sigma S-7388) was used for all of the proteases for pH-activity and -stability profiles, whereas Protazyme AK tablets were used for the temperature profiles).

An overview of the activity optima (pH- and temperature activity) is seen in Table 6. pH-stability, pH-activity and temperature-activity profiles are seen in figures 4-6, and a detailed comparison of the pH-stability data for the proteases at acidic pH-values is seen in Table 7.

Table 6

pH- and temperature optima of various proteases

Protease	pH-optimum	Temperature-optimum (°C)
Bacillus alcalophilus NCIMB 10438	9	70
Fusarium oxysporum IFO 4471	11	60
Paecilomyces lilacinus CBS 102449	8	60
Aspergillus sp. CBS 102448	10	60
Acremonium chrysogenum ATCC 48272	9	70
Acremonium kiliense ATCC 20338	11	70

Table 7

pH-stability of various proteases, between pH 2.0 and 5.0

Protease \ pH	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Bacillus alcalophilus NCIMB 10438	0.007	0.005	0.175	0.844	0.965	1.017	1.038
Fusarium oxysporum IFO 4471	0.000	0.000	0.003	0.649	0.929	1.030	1.056
Paecilomycee lilacinus CBS 102449	0.002	0.003	0.005	0.450	0.897	1.000	0.947
Aspergillus sp. CBS 102448	0.002	0.002	0.002	0.532	0.860	0.970	0.976
Acremonium chrysogenum ATCC 48272	0.002	0.001	0.001	0.809	0.894	0.972	1.005
Acremonium kiliense ATCC 20338	0.008	0.003	0.023	0.412	0.843	0.955	1.009

Example 8

Inhibition of proteases with Streptomyces Subtilisin Inhibitor (SSI)

pNA substrate: Suc-AAPF-pNA (Sigma® S-7388) was used for measuring residual activity after inhibition.

Assay buffer: 100mM succinic acid (Merck 1.00682), 100mM HEPES (Sigma H-3375), 100mM CHES (Sigma C-2885), 100mM CABS (Sigma C-5580), 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 9.0.

Assay temperature: 25°C.

SSI was purified from a *Streptomyces albogriseolus* FERM P-1205 (S-3253) fermentation supernatant by chromatography. The used SSI preparation had a purity above 95% - the purity was determined by the procedure described in Example 2A. Alternatively, SSI can be obtained from Wako in Japan, catalog no. 303-05201, manufactured by Daiwa Kasei K.K. (see eg: http://search.wako-chem.co.jp/lifedb_e/lifedocs_e/44834.asp).

Before the below inhibition assay, SSI was diluted in 0.01% Triton X-100 to A_{280} concentration = 0.010.

Protease: The used protease had a purity above 95% - the purity was determined by the procedure described in Example 2A. Before the below inhibition assay, the protease was diluted in 0.01% Triton X-100 to A_{280} concentration = 0.010.

The inhibition of the proteases by the Streptomyces Subtilisin Inhibitor (SSI) was determined by the following procedure:

A 300µl protease sample (A_{280} concentration = 0.010) was mixed with 300µl SSI (A_{280} concentration = 0.010) and 1.5 ml Assay buffer. After 15 minutes incubation at room temperature, the residual activity was measured by adding 1.5ml pNA substrate (50mg dissolved in 1.0ml DMSO and further diluted 45x with 0.01% Triton® X-100) and, after mixing, the increase in A_{405} was monitored by a spectrophotometer. As a control (no SSI), 300µl 0.01% Triton X-100 was used instead of SSI.

The residual activity was normalized with the control activity (no SSI), i.e. no inhibition by SSI will give 100% residual activity and full inhibition by SSI will give 0% residual activity.

The following results were obtained:

Protease, subtilisin from	Residual activity (%)
Bacillus sp., NCIMB 40484	4.3
Bacillus amyloliquefaciens	0.1
Bacillus amyloliquefaciens (Y217L)	0.0
Bacillus clausii, (Savinase®)	0.0
Bacillus alcalophilus, NCIMB 10438	0.0
Fusarium oxysporum IFO 4471	0.1
Paecilomyces lilacinus, CBS 102449	0.1
Aspergillus sp., CBS 102448	0.1
Acremonium chrysogenum, ATCC 48272	0.1
Acremonium kiliense, ATCC 20338	n.d.*

* not determined

Example 9

Ability of further acid-stable subtilisins to degrade insoluble parts of Soy Bean Meal (SBM)

The further acid-stable subtilisins prepared as described in Example 6 were tested as described in Example 3 for their ability to make the insoluble/indigestible parts of SBM accessible to digestive enzymes and/or added exogeneous enzymes.

The results obtained are shown in Table 8 below. For comparison, the results obtained in Example 3 for proteases I and II are included also in Table 8.

Table 8

Ability of further proteases to degrade soy remnant

Protease, subtilisin from	FITC /(+/-20000)
Bacillus alcalophilus NCIMB 10438	81300
Fusarium oxysporum IFO 4471	102200
Paecilomyces lilacinus CBS 102449	98700
Aspergillus sp. CBS 102448	123400
Acremonium chryeogenum ATCC 48272	89600
Acremonium kiliense ATCC 20338	94600
Protease I	-9200
Protease II	-1200

Example 10

Effects of the acid-stable subtilisin derived from Bacillus sp. NCIMB 40484 on the growth performance of broiler chickens

The trial was carried out at the Roche Research Center for Animal Nutrition (CRNA, F-68305 Village-Neuf, France) in accordance with the official French instructions for experiments with live animals. Day-old broiler chickens ('Ross PM3'), separated by sex, were supplied by a commercial hatchery.

The chickens were housed in wire-floored battery cages, which were kept in an environmentally controlled room. Feed and tap water was provided *ad libitum*.

On day 8, the chickens were divided by weight into groups of 6 birds, which were allocated to either the control treatment, receiving the experimental diet without enzymes, or to the enzyme treatment, receiving the experimental diet supplemented with 100 mg enzyme protein of the Bacillus sp. NCIMB 40484 protease per kg feed.

Each treatment was replicated with 12 groups, 6 groups of each sex. The groups were weighed on days 8 and 29. The feed consumption of the intermediate period was determined and body weight gain and feed conversion ratio were calculated.

The experimental diet based on maize starch and soybean meal (44 % crude protein) as main ingredients (Table 9) was produced in the CRNA. The feed was pelleted (die configuration: 3 x 20 mm) at about 70°C. An appropriate amount of the Bacillus sp. NCIMB 40484 protease was diluted in a fixed quantity of water and sprayed onto the pelleted feed. For the control treatment, adequate amounts of water were used to handle the treatments in the same way.

For the statistical evaluation, a two factorial analysis of variance (factors: treatment and sex) was carried out, using the GLM procedure of the SAS package (SAS Institute Inc., 1985). Where significant treatments effects ($p < 0.05$) were indicated, the differences between treatment means were analyzed with the Duncan test. Due to technical reasons, one cage of the enzyme treatment was excluded from the statistical evaluation.

In Table 2 the results of the growth performance of the broiler chickens from day 8 to day 29 are listed. There were no interactions between treatment and sex, therefore the pooled results of both sexes are presented. The supplementation of the experimental diet with Bacillus sp. NCIMB 40484 protease improved weight gain numerically by 6.6 %. The addition of the protease increased the feed intake slightly by 3.1 %. Bacillus sp. NCIMB 40484 protease improved the feed conversion of the broiler chickens significantly by 3.4 %.

Taking into consideration that maize starch is a highly digestible ingredient, it can be assumed that the observed effects were mainly due to the action of the enzymes on the soybean meal. Therefore, the results indicated that the nutritive value of the soybean meal was improved by the Bacillus sp. NCIMB 40484 protease.

In conclusion, the study demonstrated that supplementation of broiler feed containing high amounts of soybean meal with the Bacillus sp. NCIMB 40484 protease at 100 mg enzyme protein / kg feed resulted in a numerical increase of weight gain and a significant improvement of feed conversion.

References

EEC (1986): Directive de la Commission du 9 avril 1986 fixant la méthode de calcul de la valeur énérgetique des aliments composés destinés à la volaille. Journal Officiel des Communautés Européennes, L130, 53 - 54

SAS Institute Inc. (1985): SAS® User's Guide, Version 5 Edition. Cary NC

Table 9

Composition of the experimental diet

Ingredients (%):	
Maize starch	45.80
Soybean meal 44 [1]	44.40
Tallow	3.20
Soybean oil	1.00
DL-Methionine	0.18
MCP	0.76
Salt	0.05
Binder	1.00
Vitamin and mineral premix	3.55
Avatec® 15% CC [2]	0.06
Analyzed content:	
Crude protein (%)	19.3
ME, N-corrected (MJ/kg) [3]	12.2
Crude fat (%)	5.3

[1] analyzed content: 90.6% dry matter, 45.3% crude protein, 2.0% crude fat, 4.9% crude fibre
[2] corresponded to 90 mg lasalocid-Na / kg feed as anticoccidial
[3] calculated on the basis of analyzed nutrients content (EC-equation; EEC, 1986)

Supplier of feed ingredients

Maize starch: Roquettes Frères, F-62136 Lestrem, France
Soybean meal 44: Rekasan GmbH, D-07338 Kaulsdorf, Germany
Tallow: Fondoirs Gachot SA, F-67100 Strasbourg, France
Soybean oil: Ewoco Sarl, F-68970 Guemar, France
DL-Methionine: Produit Roche SA, F-92521 Neuilly-sur-Seine, France
MCP: Brenntag Lorraine, F-54200 Toul, France
Salt: Minoterie Moderne, F-68560 Hirsingue, France
Binder: Minoterie Moderne, F-68560 Hirsingue, France
Premix (AM vol chair NS 4231): Agrobase, F-01007 Bourg-en-Bresse, France
Avatec: Produit Roche SA, F-92521 Neuilly-sur-Seine, France

Table 10

Performance of broiler chickens from days 8 to 29

Pooled results of both sexes; mean ± st.dev.

Product	Control	Bacillus sp. NCIMB 40484 protease
Dose per kg feed	0	100 mg enzyme protein
Cages x birds	12 x 6	11 x 6 [1]
Weight gain (g/bird)	1155 [A] ± 94	1231 [A] ± 98
(%)	*100.0*	*106.6*
Feed intake (g/bird)	1941 [A] ± 108	2002 [A] ± 145
(%)	*100.0*	*103.1*
Feed conversion (g feed/g gain)	1.684 [A] ± 0.069	1.627 [B] ± 0.031
(%)	*100.0*	*96.6*

Means within a row, not sharing a common superscript are significantly different ($p < 0.05$)

[1] Due to technical reasons, one cage was excluded from the statistical evaluation

Example 11

Premix and diets for turkey and salmonids supplemented with acid-stable subtilisin protease.

A premix of the following composition is prepared (content per kilo):

5000000	IE	Vitamin A
1000000	IE	Vitamin D3
13333	mg	Vitamin E
1000	mg	Vitamin K3
750	mg	Vitamin B1
2500	mg	Vitamin B2
1500	mg	Vitamin B6
7666	mg	Vitamin B12
12333	mg	Niacin
33333	mg	Biotin
300	mg	Folic Acid
3000	mg	Ca-D-Panthothenate
1666	mg	Cu
16666	mg	Fe
16666	mg	Zn
23333	mg	Mn
133	mg	Co
66	mg	I
66	mg	Se
5.8	%	Calcium

To this premix is added Bacillus sp. NCIMB 40484 protease prepared as described in Example 2 in an amount corresponding to 10 g protease enzyme protein/kg.

Pelleted turkey starter and grower diets with a composition as shown in the below table (on the basis of Leeson and Summers, 1997 but recalculated without meat meal by using the AGROSOFT®, optimisation program) and with 100 mg protease enzyme protein per kg are prepared as follows:

Milled maize, Soybean meal, Fish-meal and Vegetable fat are mixed in a cascade mixer. Limestone, calcium phosphate and salt are added, together with the above premix in an amount of

10 g/kg diet, followed by mixing. The resulting mixture is pelleted (steam conditioning followed by the pelleting step).

Ingredient	Starter diet, g/kg	Grower, g/kg	Finisher
Maize	454.4	612.5	781.0
Soybean meal	391	279	61.7
Fish meal	70	29.9	70
Vegetable fat	21	21	46
Limestone	19	16.9	9
Calcium phosphate	30	25.9	16.8
Salt (NaCl)	2	2	2
Vitamin and mineral premix	10	10	10
Lysine	1.3	1.49	
Methionine	1.3	1.3	3.6
Calculated nutrients			
Crude protein g/kg	279	213	152
Metabolisable energy MJ/kg	12.3	12.7	14.1
Calcium, g/kg	15.8	12.7	9
Available Phosphorus, g/kg	8.2	6.4	4.6
Lysine, g/kg	17.6	12.8	7.5
Methionine, g/kg	6.1	4.9	6.9

Two diets for Salmonids are also prepared, as generally outlined above. The actual compositions are indicated in the Table below (compiled from Refstie et al (1998), Aquaculture, vol. 162, p.301-302). The estimated nutrient content is recalculated by using the Agrosoft® feed optimisation program.

The protease derived from Bacillus sp. NCIMB 40484, prepared as described in Example 2, is added to the diets in an amount corresponding to 100 mg protease enzyme protein per kg.

Ingredient	Conventional diet with fish meal	Alternative diet with soybean meal
Wheat	245.3	75.2
Fish meal	505.0	310.0
Soybean meal	-	339.0
Fish oil	185.0	200.0
DL-Methionine	13.9	23.0
Mono-Calcium phosphate	-	2.0
Vitamin and Mineral premix + pellet binder and astaxanthin	50.8	50.8
Calculated nutrients (fresh weight basis)		
Crude protein g/kg	401	415
Crude fat g/kg	232	247
Metabolisable energy MJ/kg	16.9	16.5
Calcium, g/kg	13.9	9.8
Phosphorus, g/kg	10.8	9.0
Lysine, g/kg	27.7	26.7
Methionine, g/kg	24.4	31.6

Example 12

Determination of purity of protease-containing enzyme products

The purity of protease-containing enzyme products, e.g. protease preparations such as commercial multi-component enzyme products, can be determined by a method based on the fractionation of the protease-containing enzyme product on a size-exclusion column. Size-exclusion chromatography, also known as gel filtration chromatography, is based on a porous gel matrix (packed in a column) with a distribution of pore sizes comparable in size to the protein molecules to be separated. Relatively small protein molecules can diffuse into the gel from the surrounding solution, whereas larger molecules will be prevented by their size from diffusing into the gel to the same degree. As a result, protein molecules are

separated according to their size with larger molecules eluting from the column before smaller ones.

Protein concentration assay.

The protein concentration in protease-containing enzyme products is determined with a BCA protein assay kit from PIERCE (identical to PIERCE cat. No.23225). The sodium salt of Bicinchoninic acid (BCA) is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ions (Cu^{1+}) in an alkaline environment. The BCA reagent forms the basis of the BCA protein assay kit capable of monitoring cuprous ions produced in the reaction of protein with alkaline Cu^{2+} (Biuret reaction). The colour produced from this reaction is stable and increases in a proportional fashion with increasing protein concentrations (Smith, P.K., et al. (1985), Analytical Biochemistry, vol. 150, pp. 76-85). The BCA working solution is made by mixing 50 parts of reagent A with 1 part reagent B (Reagent A is PIERCE cat. No. 23223, contains BCA and tartrate in an alkaline carbonate buffer; reagent B is PIERCE cat. No. 23224, contains 4% $CuSO_4*5H_2O$). 300µl sample is mixed with 3.0ml BCA working solution. After 30 minutes at 37°C, the sample is cooled to room temperature and A_{990} is read as a measure of the protein concentration in the sample. Dilutions of Bovine serum albumin (PIERCE cat. No. 23209) are included in the assay as a standard.

Sample pre-treatment.

If the protease-containing enzyme product is a solid, the product is first dissolved/suspended in 20 volumes of 100mM H_3BO_3, 10mM 3,3'-dimethylglutaric acid, 2mM $CaCl_2$, pH 6 (Buffer A) for at least 15 minutes at 5°C, and if the enzyme at this stage is a suspension, the suspension is filtered through a 0.45µ filter to give a clear solution. The solution is from this point treated as a liquid protease-containing enzyme product.

If the protease-containing enzyme product is a liquid, the product is first dialysed in a 6-8000 Da cut-off SpectraPor dialysis tube (cat.no. 132 670 from Spectrum Medical Industries) against 100 volumes of Buffer A + 150mM

NaCl (Buffer B) for at least 5 hours at 5°C, to remove formulation chemicals that could give liquid protease-containing enzyme products a high viscosity, which is detrimental to the size-exclusion chromatography.

The dialysed protease-containing enzyme product is filtered through a 0.45µ filter if a precipitate was formed during the dialysis. The protein concentration in the dialysed enzyme product is determined with the above described protein concentration assay and the enzyme product is diluted with Buffer B, to give a sample ready for size-exclusion chromatography with a protein concentration of 5 mg/ml. If the enzyme product has a lower than 5 mg/ml protein concentration after dialysis, it is used as is.

Size-exclusion chromatography.

A 300ml HiLoad26/60 Superdex75pg column (Amersham Pharmacia Biotech) is equilibrated in Buffer B (Flow: 1ml/min). 1.0ml of the protease-containing enzyme sample is applied to the column and the column is eluted with Buffer B (Flow: 1ml/min). 2.0ml fractions are collected from the outlet of the column, until all of the applied sample have eluted from the column. The collected fractions are analysed for protein content (see above Protein concentration assay) and for protease activity by appropriate assays. An example of an appropriate assay is the Suc-AAPF-pNA assay (see Example 2B). Other appropriate assays are e.g. the CPU assay (se Example 1), and the Protazyme AK assay (see Example 2D). The conditions, e.g. pH, for the protease activity assays are adjusted to measure as many proteases in the fractionated sample as possible. The conditions of the assays referred to above are examples of suitable conditions. Other suitable conditions are mentioned above in the section dealing with measurement of protease activity. A protein peak with activity in one or more of the protease assays is defined as a protease peak. The purity of a protease peak is calculated as the protein amount in the peak divided with the total protein amount in all identified protease peaks.

The purity of a protease-containing enzyme product is calculated as the amount of protein in the acid-stable

protease peak divided with the protein amount in all identified protease peaks using the above procedure.

5

CLAIMS

1. Use of at least one acid-stable protease in animal feed, wherein the protease

(i) is of the subtilisin family; and/or

(ii) has less than 10% residual activity when inhibited with SSI.

2. Use of at least one acid-stable protease in the preparation of a composition for use in animal feed, wherein the protease

(i) is of the subtilisin family; and/or

(ii) has less than 10% residual activity when inhibited with SSI.

3. The use of claim 1, wherein the dosage of the protease is 0.01-200 mg protease enzyme protein per kg feed.

4. The use of claim 2, wherein the intended dosage of the protease is 0.01-200 mg protease enzyme protein per kg feed.

5. A method for improving the nutritional value of an animal feed, wherein at least one acid-stable protease is added to the feed, and wherein the protease

(i) is of the subtilisin family; and/or

(ii) has less than 10% residual activity when inhibited with SSI.

6. An animal feed additive comprising

(a) at least one acid-stable protease; and

(b) at least one fat-soluble vitamin, and/or

(c) at least one water-soluble vitamin, and/or

(d) at least one trace mineral, and/or

(e) at least one macro mineral;

wherein the protease

(i) is of the subtilisin family; and/or

(ii) has less than 10% residual activity when inhibited with SSI.

7. The animal feed additive of claim 6, wherein the amount of the protease corresponds to an intended addition of 0.01-200 mg protease protein per kg feed.

8. The animal feed additive of any one of claims 6-7, which further comprises phytase, xylanase, galactanase, and/or beta-glucanase.

9. An animal feed composition having a crude protein content of 50-800 g/kg and comprising at least one acid-stable protease, wherein the protease

(i) is of the subtilisin family; and/or

(ii) has less than 10% residual activity when inhibited with SSI.

10. The animal feed composition of claim 9, wherein the amount of the protease is 0.01-200 mg protease protein per kg feed.

11. A method for the treatment of vegetable proteins, comprising the step of adding at least one acid-stable protease to at least one vegetable protein or protein source, wherein the protease

(i) is of the subtilisin family; and/or

(ii) has less than 10% residual activity when inhibited with SSI.

12. The method of claim 11, wherein soybean is included amongst the at least one vegetable protein source.

Fig. 1

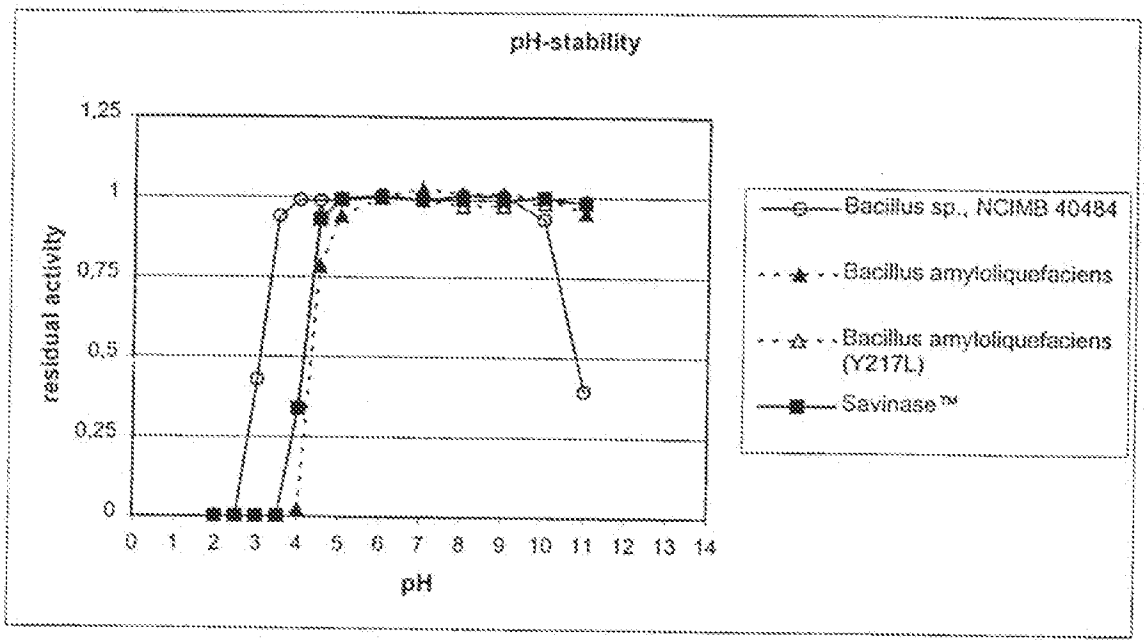

pH-stability
residual activity
1,25
1
0,75
0,5
0,25
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH
Bacillus sp., NCIMB 40484
Bacillus amyloliquefaciens
Bacillus amyloliquefaciens (Y217L)
Savinase™

Fig. 2

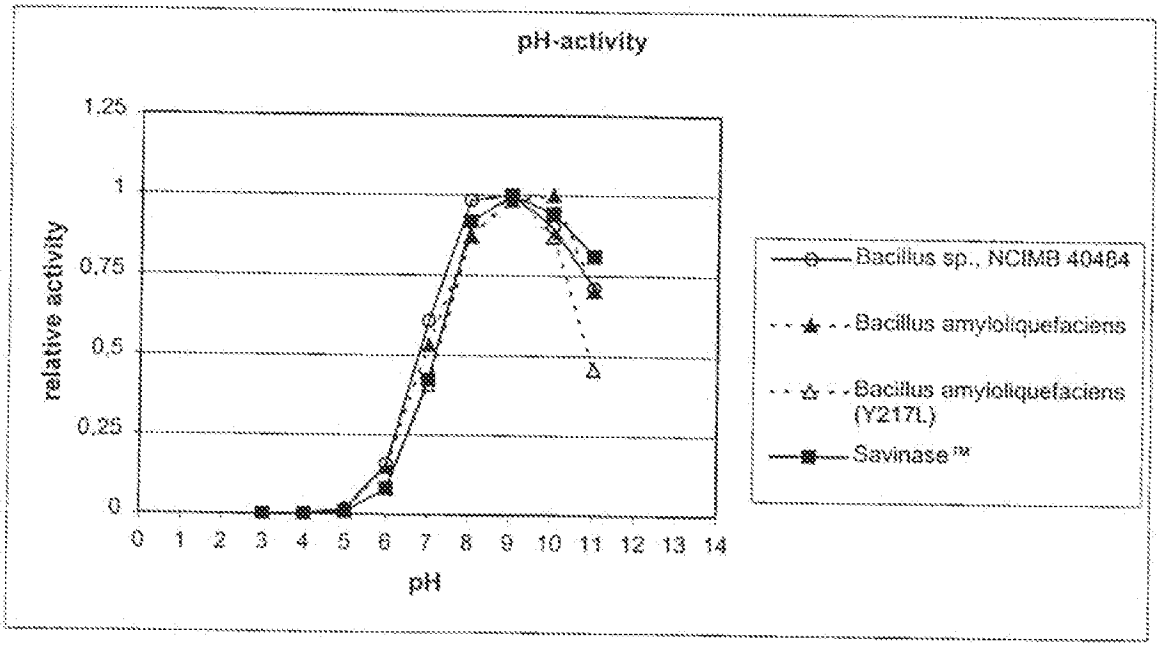

pH-activity
relative activity
1,25
1
0,75
0,5
0,25
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH
Bacillus sp., NCIMB 40484
Bacillus amyloliquefaciens
Bacillus amyloliquefaciens (Y217L)
Savinase™

Fig. 3

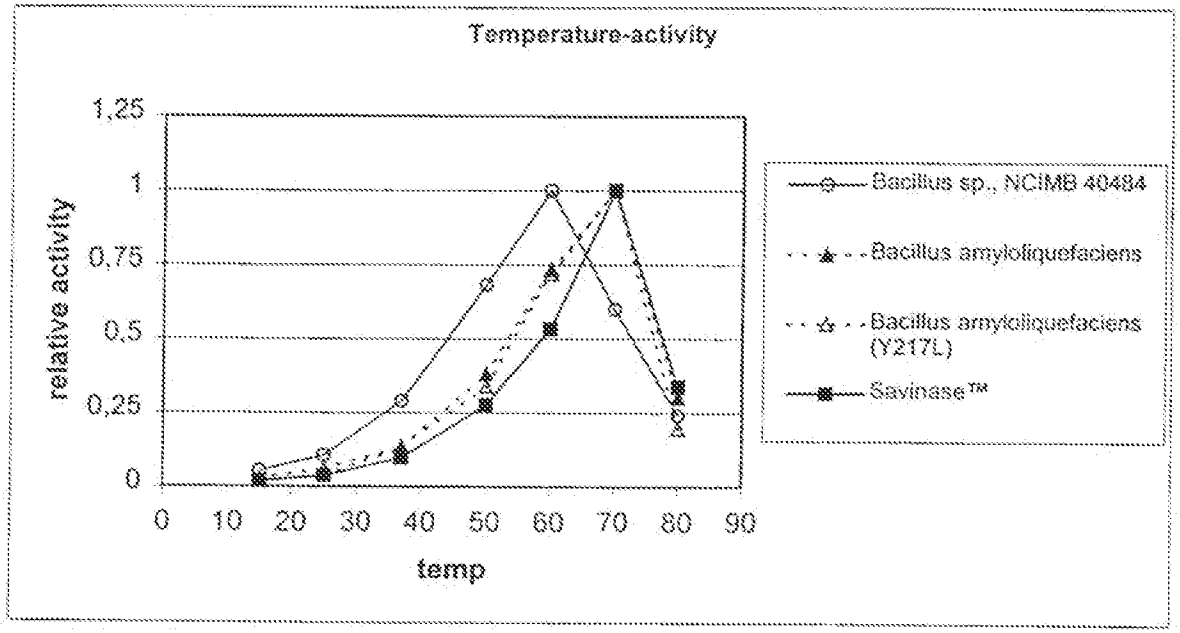

Temperature-activity
relative activity
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1
0,75
0,5
0,25
0
0 10 20 30 40 50 60 70 80 90
temp
Bacillus sp., NCIMB 40484
Bacillus amyloliquefaciens
Bacillus amyloliquefaciens (Y217L)
Savinase™

Fig. 4

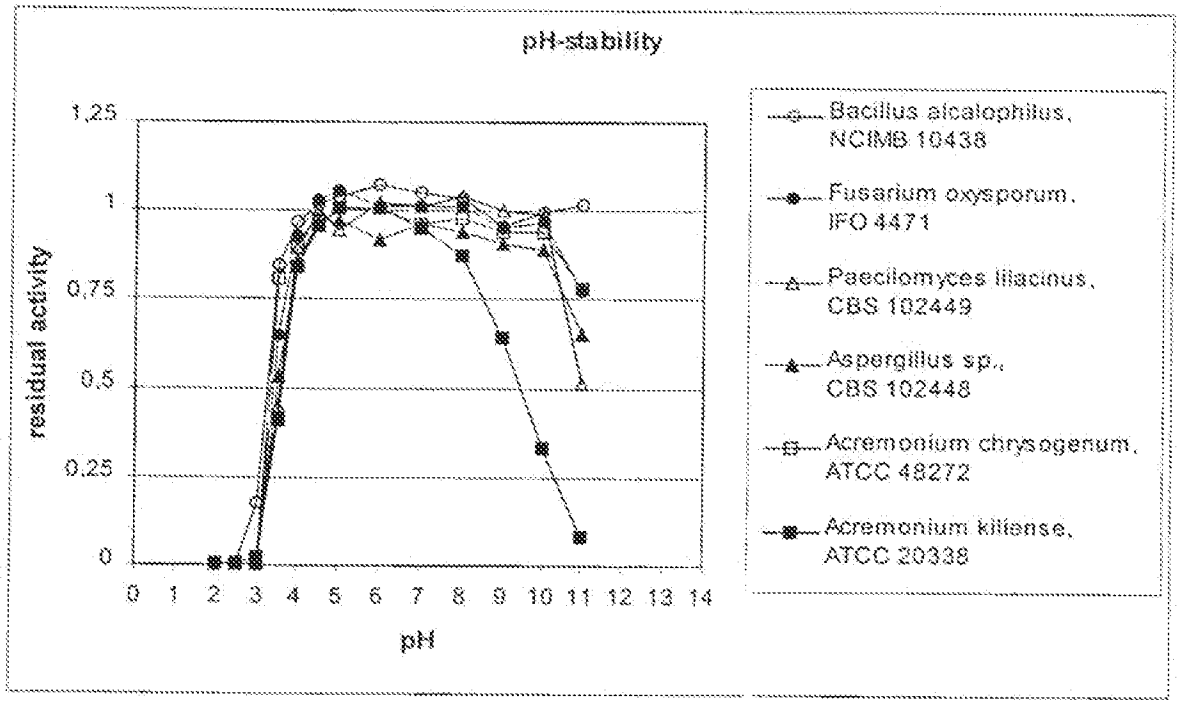

pH-stability
residual activity
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1
0,75
0,5
0,25
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH
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Fusarium oxysporum, IFO 4471
Paecilomyces lilacinus, CBS 102449
Aspergillus sp., CBS 102448
Acremonium chrysogenum, ATCC 48272
Acremonium kiliense, ATCC 20338

Fig. 5

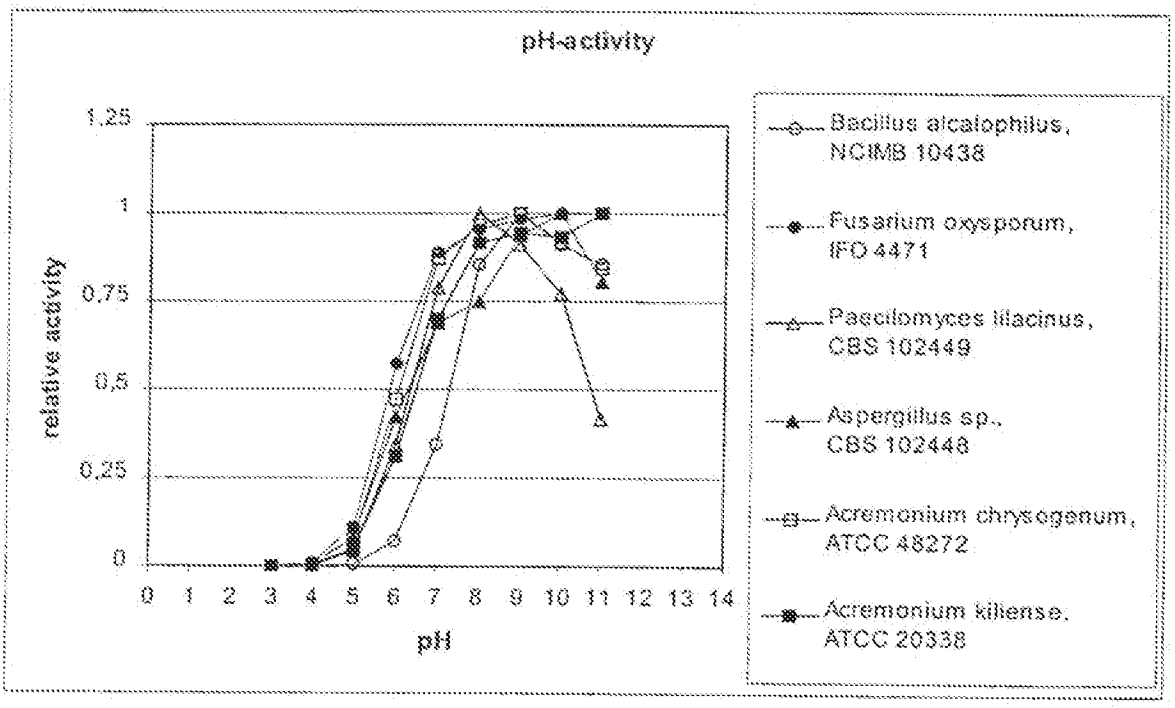
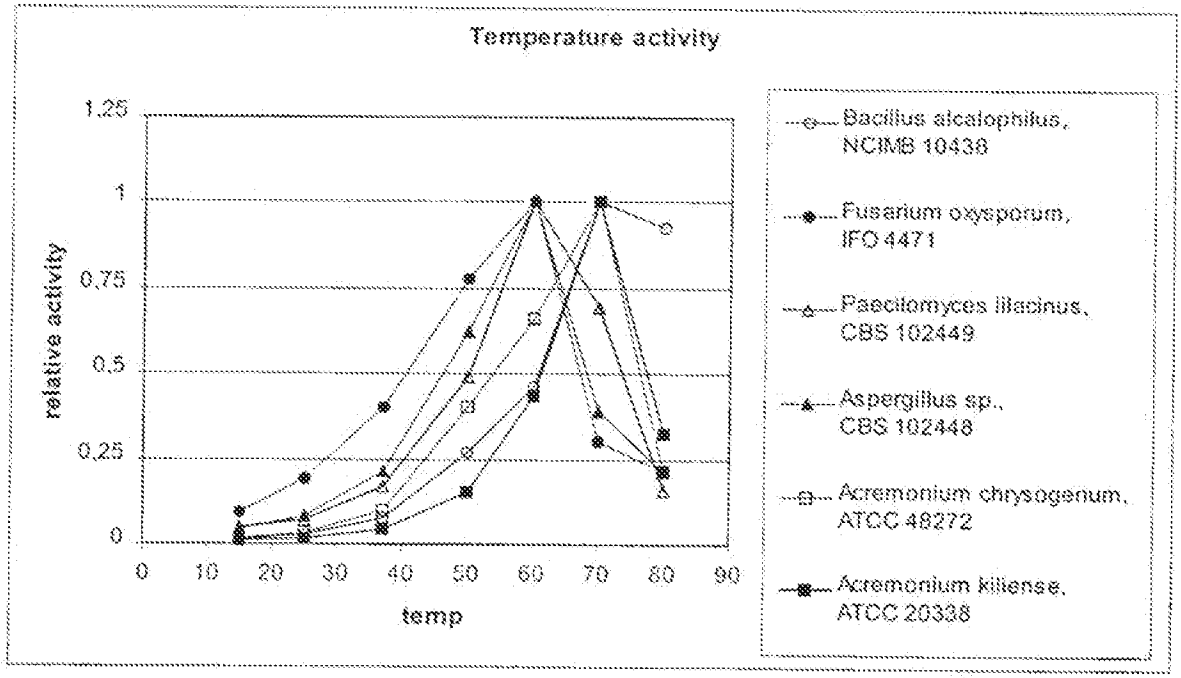

pH-activity
relative activity
1,25
1
0,75
0,5
0,25
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH
Bacillus alcalophilus, NCIMB 10438
Fusarium oxysporum, IFO 4471
Paecilomyces lilacinus, CBS 102449
Aspergillus sp., CBS 102448
Acremonium chrysogenum, ATCC 48272
Acremonium killense, ATCC 20338

Fig. 6


Temperature activity
relative activity
1,25
1
0,75
0,5
0,25
0
0 10 20 30 40 50 60 70 80 90
temp
Bacillus alcalophilus, NCIMB 10438
Fusarium oxysporum, IFO 4471
Paecilomyces lilacinus, CBS 102449
Aspergillus sp., CBS 102448
Acremonium chrysogenum, ATCC 48272
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            25                  30                  35

Gly Ser Ala Leu Asn Lys Ala Glu Ala Asn Glu Gln Lys Ala Ser Ala
        40                  45                  50

Lys Asp Pro Phe Gln Val Leu Glu Val Ala Asp Val Asp Gln Ala Val
    55                  60                  65

Lys Ala Leu Glu Asn Asn Pro Asn Val Glu Tyr Ala Glu Pro Asn Tyr
70                  75                  80                  85

Thr Phe Gln Ala Thr Trp Ser Pro Asn Asp Pro Tyr Tyr Ser Ala Tyr
                90                  95                  100

Gln Tyr Gly Pro Gln Asn Thr Ser Thr Pro Ala Ala Trp Asp Val Thr
            105                 110                 115

Arg Gly Ser Ser Thr Gln Thr Val Ala Val Leu Asp Ser Gly Val Asp
        120                 125                 130

Tyr Asn His Pro Asp Leu Ala Arg Lys Val Ile Lys Gly Tyr Asp Phe
    135                 140                 145

Ile Asp Arg Asp Asn Asn Pro Met Asp Leu Asn Gly His Gly Thr His
150                 155                 160                 165

Val Ala Gly Thr Val Ala Ala Asp Thr Asn Asn Gly Ile Gly Val Ala
                170                 175                 180

Gly Met Ala Pro Asp Thr Lys Ile Leu Ala Val Arg Val Leu Asp Ala
            185                 190                 195

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        200                 205                 210

Ala Asp Gln Gly Ala Lys Val Leu Asn Leu Ser Leu Gly Cys Glu Cys
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            265                 270                 275

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        280                 285                 290

Thr Ala Pro Gly Val Asn Ile Ala Ser Thr Val Pro Asn Asn Gly Tyr
    295                 300                 305

Ser Tyr Met Ser Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Leu
310                 315                 320                 325

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                330                 335                 340

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        35                  40                  45

Thr Phe Ser Glu Ala Ser Arg Asn Leu Asn Ala Asn Asp Leu Lys Thr
    50                  55                  60

Leu Arg Asp His Pro Asp Val Glu Tyr Ile Glu Gln Asp Ala Ile Ile
65                  70                  75                  80

Thr Ile Asn Ala Tyr Thr Gln Gln Pro Gly Ala Pro Trp Gly Leu Gly
                85                  90                  95

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            100                 105                 110

Ser Gly Gly Ser Gly Thr Cys Ala Tyr Val Ile Asp Thr Gly Val Glu
        115                 120                 125
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    130                 135                 140

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145                 150                 155                 160

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                165                 170                 175

Tyr Gly Val Lys Val Leu Asp Asn Ser Gly Ser Gly Ser Tyr Ser Gly
            180                 185                 190

Ile Ile Ser Gly Met Asp Phe Ala Val Gln Asp Ser Lys Ser Arg Ser
        195                 200                 205

Cys Pro Lys Gly Val Val Ala Asn Met Ser Leu Gly Gly Gly Lys Ala
    210                 215                 220

Gln Ser Val Asn Asp Gly Ala Ala Ala Met Ile Arg Ala Gly Val Phe
225                 230                 235                 240

Leu Ala Val Ala Ala Gly Asn Asp Asn Ala Asn Ala Ala Asn Tyr Ser
                245                 250                 255

Pro Ala Ser Gln Pro Thr Val Cys Thr Val Gly Ala Thr Thr Ser Ser
            260                 265                 270

Asp Ala Arg Ser Ser Phe Ser Asn Tyr Gly Asn Leu Val Asp Ile Phe
        275                 280                 285

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    290                 295                 300

Thr Ile Ser Gly Thr Ser Met Ala Thr Pro His Ile Val Gly Leu Gly
305                 310                 315                 320

Ala Tyr Leu Ala Gly Leu Glu Gly Phe Pro Gly Ala Gln Ala Leu Cys
                325                 330                 335

Lys Arg Ile Gln Thr Leu Ser Thr Lys Asn Val Leu Thr Gly Ile Pro
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Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Val Ser
        35                  40                  45
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    50                  55                  60

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65                  70                  75                  80

Gly Val Ala Pro Asn Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95

Asn Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Gln Trp Thr
            100                 105                 110

Ala Gln Asn Asn Ile His Val Ala Asn Leu Ser Leu Gly Ser Pro Val
        115                 120                 125

Gly Ser Gln Thr Leu Glu Leu Ala Val Asn Gln Ala Thr Asn Ala Gly
    130                 135                 140

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145                 150                 155                 160

Tyr Pro Ala Arg Tyr Ala Asn Ala Leu Ala Val Gly Ala Thr Asp Gln
                165                 170                 175

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            180                 185                 190

Val Ala Pro Gly Val Gly Ile Gln Ser Thr Tyr Pro Gly Asn Arg Tyr
        195                 200                 205

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    210                 215                 220

Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Thr Gln Ile
225                 230                 235                 240

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                245                 250                 255

Phe Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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